Short Communication:

Identification of the heterotrophic nanoflagellate *Bilabrum latius* in the southern Adriatic (Mediterranean Sea)

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Abstract

Heterotrophic flagellates (HF) represent an important protist group in marine ecosystem functioning. Characterised by high taxonomic diversity, identification and classification of HF is often difficult using classical methods of light microscopy (LM). Complementing LM with molecular methods, such as barcoding, enables reliable taxonomic identification of even small size nanoflagellates that share similar or unnoticeable morphological features. The order Bicosoecida is a group of heterotrophic nanoflagellates that are important part of protist plankton and benthic communities of the world oceans. Recently, on the basis of high-resolution light microscopy and barcoding, a new bicosoecid genus, *Bilabrum*, was described with *B. latius* sp. as a type species. Our study reports on identification of *B. latius* from co-culture with the diatom species *Chaetoceros affinis* isolated from fresh plankton samples collected in the southern Adriatic. This detection of the Adriatic *B.latius* represents first record

of this species outside its´ up to now known and described habitat (deep-sea sediment of the South - East Atlantic Ocean) and in diatom co-culture.

Keywords:

heterotrophic nanoflagellate, Bicosoecida, Adriatic Sea, plankton, barcoding, light microscopy

Introduction

In the marine microbial community, unicellular heterotrophic flagellates (HFs) represent a well-known group of bacteria feeders (Azam et al., 1983; Boenigk and Arndt, 2002). As the main predators of prokaryotes, in the marine environment, HFs play an important role in the biogeochemical cycles connecting dissolved organic matter through prokaryotic grazing to higher trophic levels (De Corte et al., 2019; Hahn and Höfle, 1999). Parasitism and herbivory by HFs also play an important role within the marine food net (Kühn and Hofmann, 1999; Sherr and Sherr, 1994; Weisse et al., 2016). Moving and feeding with flagella and cells without chloroplasts are common features of this extremely diverse group of protists. Generally, HFs are found in a size range between 1 - 30 µm, with species smaller than 15 µm classified as heterotrophic nanoflagellates (HNFs) (Arndt et al., 2000). HF are also characterised by a different ecology and are adapted to a variety of habitats; from benthic to pelagic, coastal and oceanic ecosystems(Cavalier-Smith and Chao, 2006; Hohlfeld and Arndt, 2022; Schoenle et al., 2021, 2020). Dominant taxonomic groups of HF include stramenopile taxa, dinoflagellates, choanoflagellates, kathablepharids, euglenids, free-living kinetoplastids and cercozoans (Arndt et al., 2000). Among stramenopile taxa, the order Bicosoecida (bicosoecids) is characterised by a small cell size (< 8 µm), and species free-living as plankton or attached to substrata by the posterior flagellum or stalk. Being small in size, bicosoecids are difficult to identify using microscopy and barcoding is a valuable tool to overcome that limitation. Recently, a detailed phylogenetic reconstruction of bicosoecids, based on high-resolution light microscopy and barcoding, enabled the erection of a new genus, *Bilabrum,* Schoenle & Arndt, 2020, with *B. latius* Schoenle & Arndt, 2020, as type species (Schoenle et al., 2020). This new genus and type species was first characterised as the *Cafeteria* genus strain isolated from deep-sea sediment of the Angola Basin (south east Atlantic Ocean) (Arndt et al., 2000) and morphologically shared similar cell characteristics with the *Cafeteria* genus, but molecular barcoding of the 18S rDNA showed clear separation (p-distance of 10% to *Cafeteria* species) of the *Bilabrum* genus as a separate group in the bicosoecids phylogeny. Bicosoecid species are most commonly studied and identified by isolation and cultivation of single cells, followed by molecular barcoding (Scheckenbach et al., 2005; Weber, 2017). While some genera (e.g. *Cafeteria*) grow opportunistically under laboratory conditions, others are less easy to culture and remain undescribed (del Campo et al., 2013). Awareness of the ecological diversity and abundance of these uncultivated bicosoecids is available thanks to sequencing data obtained through metabarcoding studies (de Vargas et al., 2015; del Campo and Massana, 2011; Schoenle et al., 2021; Stecher et al., 2015). Co-occurrence and successful culture growth of more than one HF species as well as HF species with other protists such as diatoms has been recorded (Andersen, 2005; Arndt et al., 2000). Genus *Chaetoceros* (Ehrenberg) is one of the most abundant and species rich diatom genera in marine plankton worldwide, Adriatic Sea included (De Luca et al., 2019; Viličić et al., 2009). In taxonomy and ecology studies *Chaetoceros* species have been successfully isolated and cultivated in laboratory conditions (Gaonkar et al., 2017; Tanković et al., 2018). *Chaetoceros affinis* is a plankton species with cosmopolitan distribution. *C. affinis* cells united in chains and morphologically characterised with large terminal setae (Hasle and Syvertsen, 1996).

Previous research on HFs in the Adriatic Sea mainly includes studies on abundance and distribution (Fuks et al., 2012; Šolić et al., 1998) of this protist group as a whole, influenced by environmental conditions, with little data on taxonomic composition or species identity. Diversity, ecology and distribution patterns of bicosoecids are not described for the Adriatic.

In our study, we describe for the first time the occurrence of *B. latius* outside its original description habitat, found in the plankton realm of the Adriatic Sea. Furthermore, we report for the first time an unexpected interaction between *B. latius* and diatom species *C. affinis* from plankton samples collected in the southern Adriatic Sea.

Methods

Sampling and cultivation

Phytoplankton sampling was carried out in March 2022 for the national grant project on the molecular life strategies of Adriatic phytoplankton (AdriLife). On the southern Adriatic station (**Figure 1**, Lokrum station, gps coordinates: 42°37'21.0"N, 18°06'00.0"E), positioned in the Dubrovnik city coastal area, phytoplankton was collected with a vertical net haul from bottom to surface (depth = 90 m). From a collected net sample, monoclonal culture of *Chaetoceros affinis* diatom species was established by isolating one *C. affinis* cell in f/2 medium (Guillard, 1975)and growth under conditions of 12:12 h light/dark photoperiod, 16 °C, and 75 μmol photons $m^{-2}s^{-1}$ as a batch culture number CIM1041 at the Center for Marine Research (Rovinj, Croatia) culture collection. After three weeks of growth, a nanoflagellate species was confirmed as a co-culture of *C. affinis.*

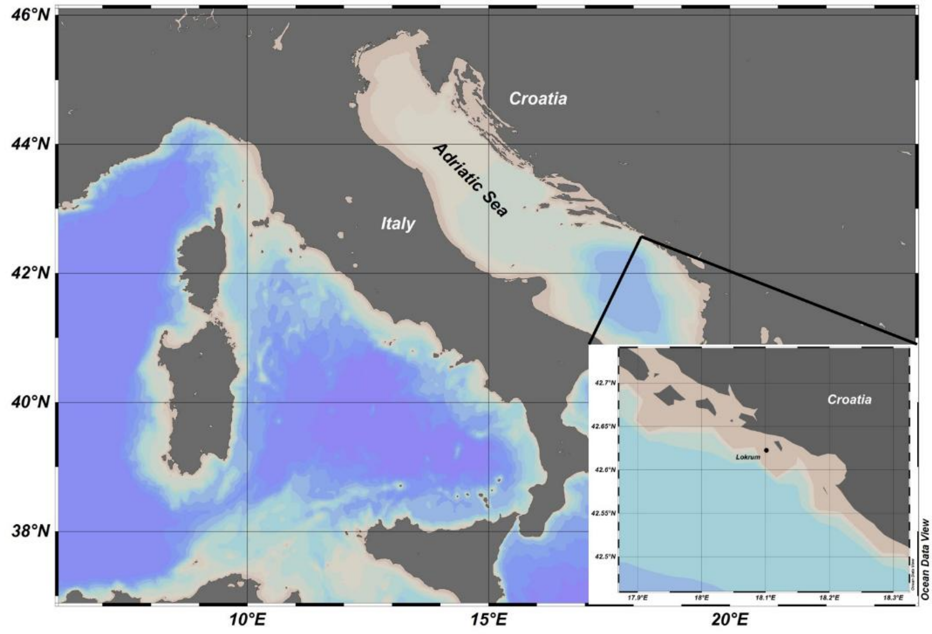


Figure 1 Map of the Adriatic Sea and the location of the study sampling station (Lokrum station)

Light microscopy (LM)

An inverted light microscope (Axio Observer , Zeiss, Germany) equipped with phase contrast, Nomarski differential interference contrast and bright field optics was used for the morphological characterisation of *C. affinis* and nanoflagellate species. Observations and cell measurements (cell length, width and flagella characterisation) were performed on CIM1041 culture with the oil objective EC Plan-NEOFLUAR 100x/1.3 Oil objective. Pictures and video were taken using an Axiocam305 mono digital camera and analysed in Zen 3.4. Software (Zeiss, Germany).

Molecular analysis (DNA barcoding and phylogeny)

For barcoding, 150 mL of *C. affinis* strain CIM1041 batch culture (*C. affinis* $4.01x10^4$ cells/mL) was pre-filtered on 3 μm pore size mixed cellulose membrane (Whatman) to separate *C.affinis* cells from the culture and the filtrate was further processed on 0.2 μm pore size mixed cellulose membrane (Whatman, United States). DNeasy® Plant Mini Kit (Qiagen, United States) according to manufacturer instructions was used for genomic DNA (gDNA) isolation of nanoflagellate cells from the 0.2 µm filter.gDNA was eluted in 50 µL elution buffer and the quality and quantity was checked on the nanodrop (NanoPhotometer, Implen). Isolated gDNA was used as a template for PCR amplification of the barcoded regions for 18S and 28S RNA genes. Reaction mixture (25 µL) contained 200 µM of each dNTP, 0.3 µM of each primer, 4 mM MgCl2, 1× DreamTaq Green buffer, 0.2 U of DreamTaq DNA polymerase (Thermo Fisher Scientific) and 5 ng of genomic DNA. PCR conditions were as follows: an initial denaturation step of 5 min at 95˚C, 33 cycles of 40 s at 95˚C, 40 s at 52˚C and 1 min at 72˚C, and final extension step of 5 min at 72˚C.The primers used for amplification were F-566 (5'-CAGCAGCCGCGGTAATTCC -3') and R-1200 (5'- CCC GTGTTGAGTCAAATTAAGC-3') for 18S(Hadziavdic et al., 2014) and D1R (5’-ACCCGCTGAATTTAAGCATA-3’),D3Ca (5’-ACGAACGATTTGCACGTCAG-3’) for 28S(Bruder and Medlin, 2007; Scholin et al., 1994). The sizes of the amplified gene regions were around 600 bp and 800 bp for 18S and 28S, respectively. PCR products were purified by NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Germany) and Sanger sequenced bidirectionally using the service of Macrogen Europe (The Netherlands). For the bioinformatics analyses, Geneious software (Kearse et al., 2012) was used. Sequences were aligned using MAFFT (Katoh and Standley, 2013) and consensus (highest quality threshold – chromatogram quality to call for best base) used for BLAST search (Altschul et al., 1990) and phylogenetic analyses. Reference sequences for the phylogeny were downloaded from GenBank (Clark et al., 2016). PhyML (Guindon et al., 2010) was used to build Maximum likelihood (ML) phylogenetic trees employing GTR (General Time Reversible) model of nucleotide substitution and statistical support with 100 bootstrap replicates. MrBayes (Ronquist et al., 2012) was used for Bayesian interface (BI) phylogenetic analysis employing GTR model and a four-category gamma distribution. For the 18S phylogeny, BI analysis consisted of 50,000 generations, sample frequency of 10 and burn – in length of 1,250 (25% of samples) and for 28S BI analysis consisted of 10,000 generations, sample frequency of 10 and burn – in length of 250 (25% of samples) The barcoding sequences of the nanoflagellate CIM1041 were stored in GenBank under the Accession Numbers OR016443 and OR016445.

Metabarcoding database *Bilabrum* search

The presence of the 18S rDNA sequence of *Bilabum latius* in the publicly available metabarcoding datasets was investigated using the metaPR2 interactive website (https://shiny.metapr2.org) of the publicly available metaPR2 database of curated metabarcodes (Vaulot et al., 2022). The latest version (released in spring 2022) of metaPR2 contains 41 public datasets, including datasets from the global marine sampling expeditions Biomarks, Malaspina, Ocean Sampling Day, Tara Oceans and many Arctic datasets. The search settings for samples in the metaPR2 database were set for the V4 gene region, oceanic and coastal ecosystems, different substrates (water, biofilm, sediment and sand), all size fractions (pico - meso) and all depth levels (sub-ice, surface, euphotic, mesopelagic, bathypelagic, composite, bottom). The minimum number of total reads per ASV was set to 100 and all taxa were considered. With these settings, a total of 3251 metabarcoding samples were selected and included in the sequence search of *B. latius*. BLAST searched for ASVs similar to the query sequence. We used the 18S reference sequence of *B. latius* (MN315515) as the query. All ASVs were searched, but only those with a minimum sequence identity of 80 % were displayed as search results.

Results and Discussion

After three weeks of growth of the diatom *C. affinis* in a batch culture, the co-occurrence of nanoflagellates in the diatom culture was observed. Morphological characterisation by light microscopy revealed that the co-cultured nanoflagellate CIM1041 has a slightly elongated, globular cell shape and has two clearly visible flagella: a posterior one (2.89 ± 0.36 µm, 2.63 -

3.15 µm, n = 10), which we observed the nanoflagellate to use for attachment to *C. affinis* cells or organic cell culture particles, and an anterior one (5.332 ± 0.424 µm, 4.97 – 5.80 µm, n = 10), which is very active in feeding and swimming. The mean width of the cell was 2.388 (± 0.381) µm (1.82 – 2.74 µm, n = 10) and the mean length was 4.574 (± 0.901) µm (3.35 – 5.66 µm, n = 10). The mean size was in a similar size range (mean cell length of 2.2 – 3.5 µm, mean cell width of 1.9 – 2.9 µm, and length of both flagella about 2.4 – 6.9 µm) as described for species of the bicosoecid family *Cafeteriaceae* and *B. latius* (Schoenle et al., 2020), but reliable species identification was not possible with light microscopy. The pronounced lip (pleat-like thickenings) characteristic of *B. latius* was not visible in the nanoflagellate CIM1041 by light microscopy (**Figure 2C**).

The molecular identification of the nanoflagellate CIM1041 confirmed the species identity with *B. latius*. For both barcoding genes (18S and 28S), a BLASTn search with the non-redundant nucleotide reference database (GenBank) confirmed 100% sequence identity with the reference sequences (GenBank Acc. Number MN315515 and MN315517 for 18S and 28S, respectively) deposited as a new bicosoecid species - *Bilabrum latius* (**Figure 2**). While for 28S only one reference sequence from *Bilabrum* is available in GenBank, for the 18S gene, in addition to the reference sequence from *B. latius*, a set of 6 sequences described as uncultured eukaryote isolates from intertidal mud sediment from the Bay of Bourgneuf (French Atlantic coast) (unpublished study) shows 100% sequence identity with the 18S gene reference sequence MN315515 from *B. latius*. Phylogenetic analysis confirms that the genus *Bilabrum* is more widespread than previously known (only one record of the genus/species in the Angola Basin).

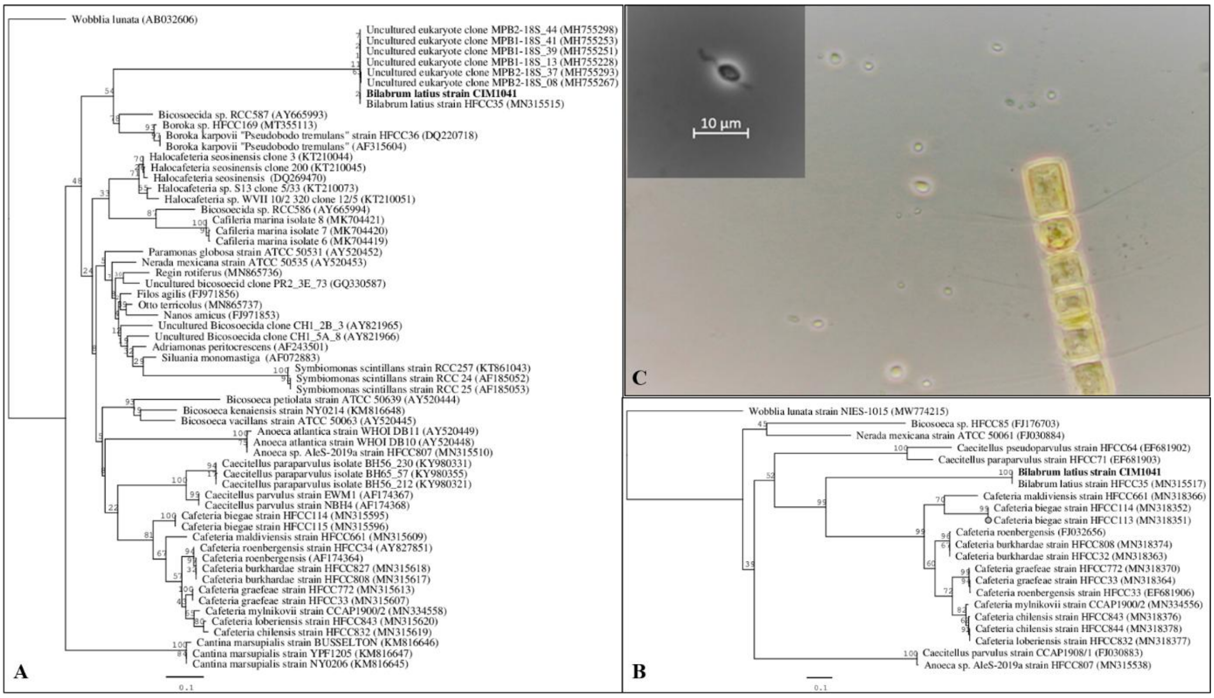
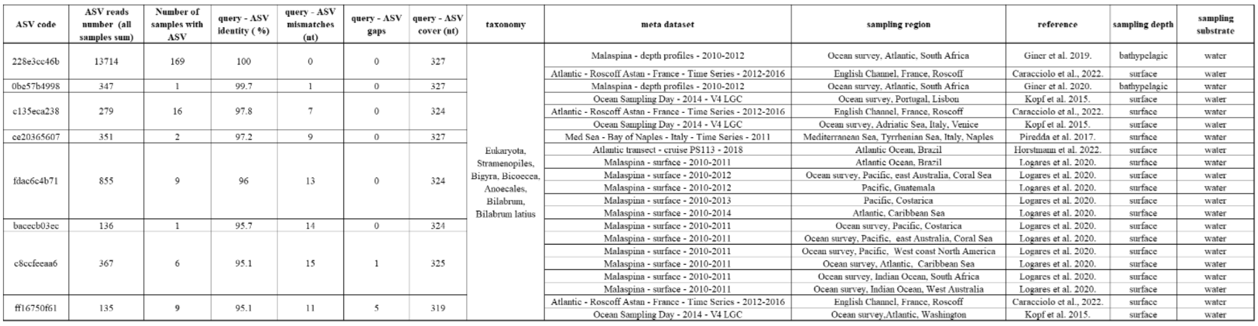


Figure 1 (**A**) 18S and (**B**) 28S gene maximum likelihood (ML) phylogenetic trees of the *Bilabrum latius* strain CIM1041 and the available GenBank reference sequences of the *Bilabru*m genus and other bicosoecids. Accession numbers of the reference sequences are indicated in parenthesis in sequence names. This study *Bilabrum latius* strain CIM1041 is marked in bold. 18S and 28S gene Bayesian interface (BI) phylogenetic trees of the *Bilabrum latius* strain CIM1041 and the available GenBank reference sequences of the *Bilabrum* and other bicosoecids are available in **Supplementary Figure 1**. (**C**) Light microscopy image of the diatom *Chaetoceros affinis* and nanoflagellate *Bilabrum latius* co-culture with magnification of a single nanoflagellate cell in the image left corner. The trees is outgroup-rooted with *Wobblia lunata* 18S and 28S reference sequences.

Furthermore, Adriatic (CIM1041) and Atlantic (MN315515) *B. latius* have the same 18S and 28S genotype, but Adriatic was cultured from the collected plankton sample, while Atlantic was from deep-sea sediment. This result is consistent with previous studies that confirmed the possibility of finding the same genotype of a heterotrophic flagellate in opposite marine habitats such as surface waters and deep-sea sediments (Scheckenbach et al., 2005; Schoenle et al., 2020). The identification of *B. latius* in the Adriatic supports the evidence for a continuous exchange between deep-sea and surface communities discussed in Schoenle et al. 2020. Identification of *B. latius* also confirmed interplay of the heterotrophic nanofllagelate and diatom in culture. Previously, small HF and diatoms in cultures and environment were mainly described through examples of parasitic interactions (Kühn and Hofmann, 1999; Schweikert and Schnepf, 1997). Observation of the *B. latius* and interaction in the studied culture indicates that *B. latius* is feeding on bacteria that probably use the organic matter exuded by diatom cells into the surrounding environment (cell culture medium). Still, further research of the *B. latius* ecology and physiology is needed to describe in detail this nanoflagellate diatom co-culture interplay and possible relevance within the marine environment.

The 18S rDNA search in the metaPR2 database enabled the recognition of *Bilabrum latius* and an insight into its global distribution before this species was isolated, cultured and taxonomically confirmed based on morphological characters identified by microscopy. With the search settings applied, a total of 8 matching ASVs (**Table 1**) were found, occurring in 6 different metabrcoding records (Caracciolo et al., 2022; Giner et al., 2020; Hörstmann et al., 2022; Kopf et al., 2015; Logares et al., 2020; Piredda et al., 2017). All matched ASVs were classified as *Bilabrum latius* and had over 95% identity with the *B. latius* query sequence.

Table 1 Results of the metaPR2 database search. ASVs with more than 80% sequence identity with the *Bilabrum latius* query sequence are listed (ASV code). For each ASV found, number of reads, samples, BLAST features, taxonomy and dataset characteristics are indicated.



ASV code	ASV reads number (all samples sum)	Number of samples with ASV	query - ASV identity (%)	query - ASV mismatches (nt)	query - ASV gaps	query - ASV cover (nt)	taxonomy	meta dataset	sampling region	reference	sampling depth	sampling substrate
228e3cc46b	13714	169	100	0	0	327	Eukaryota, Stramenopiles, Bigyra, Bicoecea, Anoecales, Bilabrum, Bilabrum latius	Malaspina - depth profiles - 2010-2012	Ocean survey, Atlantic, South Africa	Giner et al. 2019.	bathypelagic	water
								Atlantic - Roscoff Astan - France - Time Series - 2012-2016	English Channel, France, Roscoff	Caracciolo et al., 2022.	surface	water
0be57b4998	347	1	99.7	1	0	327		Malaspina - depth profiles - 2010-2012	Ocean survey, Atlantic, South Africa	Giner et al. 2020.	bathypelagic	water
c135eca238	279	16	97.8	7	0	324		Ocean Sampling Day - 2014 - V4 LGC	Ocean survey, Portugal, Lisbon	Kopf et al. 2015.	surface	water
								Atlantic - Roscoff Astan - France - Time Series - 2012-2016	English Channel, France, Roscoff	Caracciolo et al., 2022.	surface	water
								Ocean Sampling Day - 2014 - V4 LGC	Ocean survey, Adriatic Sea, Italy, Venice	Kopf et al. 2015.	surface	water
ce20365607	351	2	97.2	9	0	327		Med Sea - Bay of Naples - Italy - Time Series - 2011	Mediterranean Sea, Tyrrhenian Sea, Italy, Naples	Piredda et al. 2017.	surface	water
fdac6c4b71	855	9	96	13	0	324		Atlantic transect - cruise PS113 - 2018	Atlantic Ocean, Brazil	Horstmann et al. 2022.	surface	water
								Malaspina - surface - 2010-2011	Atlantic Ocean, Brazil	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2012	Ocean survey, Pacific, east Australia, Coral Sea	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2012	Pacific, Guatemala	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2013	Pacific, Costarica	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2014	Atlantic, Caribbean Sea	Logares et al. 2020.	surface	water
bacecb03ec	136	1	95.7	14	0	324		Malaspina - surface - 2010-2011	Ocean survey, Pacific, Costarica	Logares et al. 2020.	surface	water
c8ccfeeaa6	367	6	95.1	15	1	325		Malaspina - surface - 2010-2011	Ocean survey, Pacific, east Australia, Coral Sea	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2011	Ocean survey, Pacific, West coast North America	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2011	Ocean survey, Atlantic, Caribbean Sea	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2011	Ocean survey, Indian Ocean, South Africa	Logares et al. 2020.	surface	water
								Malaspina - surface - 2010-2011	Ocean survey, Indian Ocean, West Australia	Logares et al. 2020.	surface	water
ff16750f61	135	9	95.1	11	5	319		Atlantic - Roscoff Astan - France - Time Series - 2012-2016	English Channel, France, Roscoff	Caracciolo et al., 2022.	surface	water
								Ocean Sampling Day - 2014 - V4 LGC	Ocean survey,Atlantic, Washington	Kopf et al. 2015.	surface	water

The ASVs found were not represented by a high number of reads. The average contribution of each ASV to the total number of eukaryote reads in its dataset was less than 1% (0.32%). All ASVs were detected in water column samples and mostly in surface depth. Only two ASVs (sampling region Atlantic, South Africa) were from deep marine waters (bathypelagic). The geographical distribution of ASVs was broad and included coastal and ocean sites (sampling regions) in the Atlantic, Pacific, Indian Ocean as well as the Mediterranean Sea (**Table 1**). One ASV (ASV code: c135eca238) was identified in the Adriatic Sea (Venice Lagoon) from 2014. Ocean Sampling Day dataset identified and showed 97.8% identity with the *Bilabrum latius* query sequence. This finding confirms the presence of the *Bilabrum* species in the Adriatic Sea before 2022 and the isolation and cultivation of the study strain CIM1041 with C. affinis. Nevertheless, *Bilabrum* ASV was detected in the northern Adriatic (Venice Lagoon), while *Bilabrum latius* CIM1041 was isolated from the net sample of the southern Adriatic (Lokrum station). Also, the 97.8% sequence identity of the ASV from the Venice Lagoon suggests a different genotype of *B. latius* or even different species of the genus *Bilabrum*. Since the number of cultivated, described *Bilabrum* species and known (publicly available) *Bilabrum* sequences is low and the genus phylogeny is not clarified, the taxonomic assignment of the *Bilabrum* ASVs discovered in the metaPR2 database has yet to be confirmed. Interestingly, the ASVs (ASV code: 228e3cc46b and 0be57b4998) with the highest sequence identity (100 and 99%) with the *B. latius* query are from datasets collected in the French and African Atlantic coastal regions, where the only 18S reference sequences known to date are from *B. latius* (**Figure 2**), confirming the presence of the *B.latius* species in these regions. Research on bicosoecids is very sparse for the Adriatic and *Bilabrum* is a recently described new genus. Therefore, future ecology and metabarcoding studies in this area will allow a detailed description of the diversity and distribution of the genus *Bilabrum* in the Adriatic.

The successful detection of *Bilabrum* ASVs in previous global ocean metabarcoding datasets (metaPR2 database) indicates the worldwide distribution of this genus and defines it as a common member of the plankton community, but usually present in low abundance. Due to the low abundance and the limited possibilities of light microscopic identification of small plankton organisms without distinct (specific) morphological features, *Bilabrum* remained unnoticed in previous plankton studies.

The worldwide occurrence of *Bilabrum* confirms a link between distant marine environments through the circulation of the world's oceans and/or the sharing of a suitable habitat (plankton realm) for this heterotrophic nanoflagellate genus. Further research, especially through new intensive metabarcoding studies, will help to gain valuable insights into this small heterotrophic nanoflagellate *B. latius* and its role in the world's oceans and the Adriatic Sea. Since species-rich reference databases form the basis for metabarcoding and the characterisation of plankton communities, even small, local research and discoveries like the one in our study make an important contribution to knowledge about the biodiversity and ecology of nanoflagellate species like *B. latius*.

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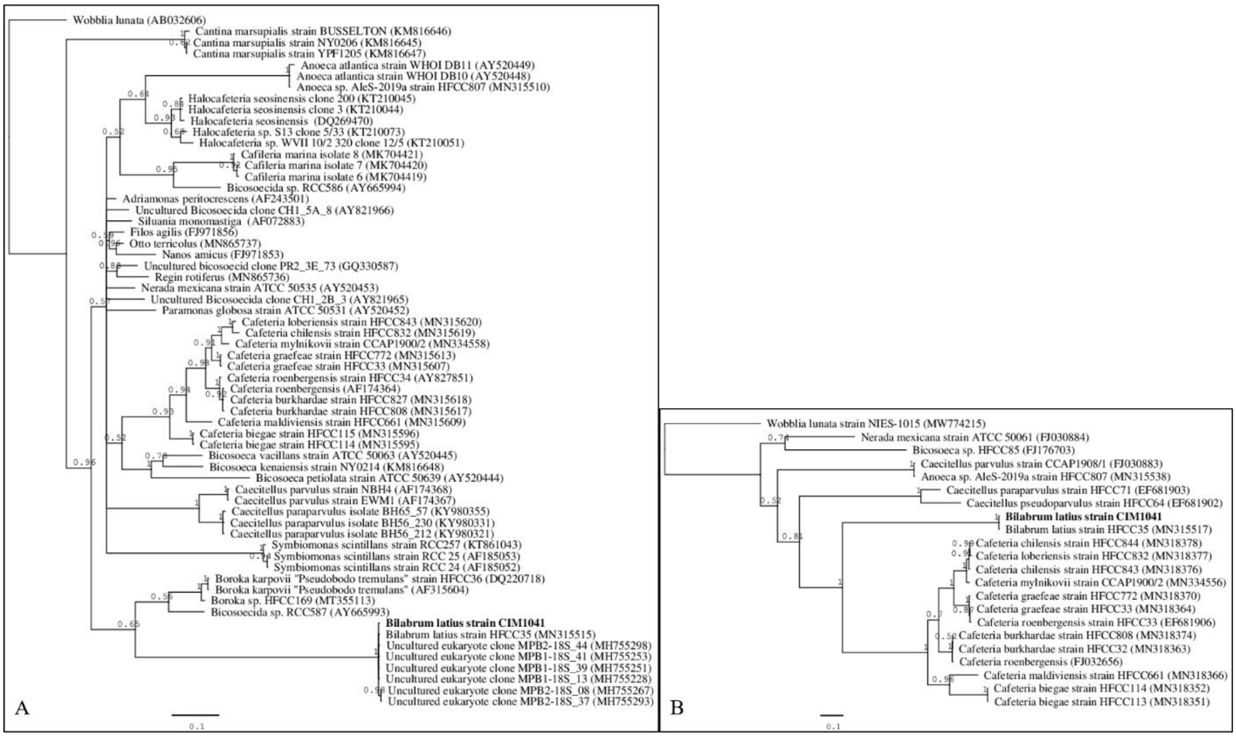
Conflicts of Interest: The authors have no competing interests to declare.



Supplementary Figure 1 (**A**)18S and (**B**) 28S gene Bayesian interface (BI) phylogenetic trees of the *Bilabrum latius* strain CIM1041 and the available GenBank reference sequences of the *Bilabrum* genus and other bicosoecids. Accession numbers of the reference sequences are indicated in parenthesis in sequence names. This study *Bilabrum latius* strain CIM1041 is marked in bold. The trees are outgroup-rooted with *Wobblia lunata* 18S and 28S reference sequences.

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