Morphology and phylogeny of picoeukaryotes and planktonic pennate diatoms in the middle and south Adriatic Sea

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Doctoral thesis / Disertacija

2018

University of Zagreb

FACULTY OF SCIENCE
DEPARTMENT OF BIOLOGY

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DOCTORAL THESIS

Zagreb, 2018

Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET

BIOLOŠKI ODSJEK

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MORFOLOGIJA I FILOGENIJA PIKOEUKARIOTA I PENATNIH PLANKTONSKIH DIJATOMEJA U SREDNJEM I JUŽNOM JADRANU

DOKTORSKI RAD

Zagreb, 2018

This doctoral dissertation was carried out as a part of the postgraduate programme at University of Zagreb, Faculty of Science, Department of Biology – Botany, under the supervision of dr.sc. Zrinka Ljubešić. The research was performed in the frame of the Bio-tracing Adriatic Water Masses (BIOTA) project, supported by the Croatian Science Foundation (project number UIP-2013-11-6433); project leader dr.sc. Zrinka Ljubešić). The experimental part of the research was carried out in part at Ruđer Bošković Institute, Zagreb, Croatia, while part of the bioinformatic analyses was carried out at University of Arkansas, Fayeteville, USA.

Acknowledgments

Firstly, I would like to express my sincere gratitude to my supervisor Dr Zrinka Ljubešić for the continuous support of my Ph.D. study and related research, for her patience, guidance, motivation, and immense knowledge. She helped me in all phases of research and writing of this thesis. I could not have imagined having a better supervisor for my Ph.D. study.

In addition to my supervisor, I would like to thank my thesis committee: Dr Daniela Marić Pfannkuchen, Dr Sunčica Bosak and Dr Regine Jahn, for their insightful comments and encouragement, but also for the hard questions, which motivated me to widen my research from various perspectives.

My sincere thanks also goes to Dr Sandi Orlić and Dr Petra Peharec Štefanić, who enabled me access to the laboratory and research facilities without which I would not be able to examine my samples. I would also like to thank my 'over the sea' colleagues and co-authors Dr Teofil Nakov, Dr Elizabeth Ruck, Dr Daniel Vaulot, Dr Romain Gastineau, Dr David G Mann and Dr Rosa Trobajo for their sincere guidance and fruitful discussions. Without they precious support it would not be possible to conduct this research.

I thank my fellow lab mates, current and previous, colleagues from abroad, students, interns and colleagues passing through the lab or cafeteria☺. Thank you Sunčica, Maja, Anamarija, Pero, Kora, Tonka, Mirela, Ana, Tihana, Tanja, Mišel, Dorotea, Zuzanna, Chetan, Solenn, Karoline, Mirta, Veronika, Nikola; for the stimulating discussions, for help, support and encouragement, and for all the fun we have had in the last four years. Additionally, I would like to thank the rest of co-authors on research publications involved in this thesis.

I would also like to thank my girls, my best friends, who were, are, or will be on the same life journey, professionally and privately, for their help, patience, listening, encouraging, laughing, partying, crying, and experiencing all the other feelings in between… Thank you Petra, thank you Marina, thank you Sany, thank you Franka, thank you Zrinka! Priatel'ce 4ever ☺

I would like to thank my family: my parents Pero and Ljiljanka, for their irreplaceable love and support, to my brother Danijel and my sisters Martina and Petra for supporting me spiritually throughout writing this thesis and my life in general.

In the end, I want to thank my husband Antonio, for his overwhelming love, support, for being my soulmate and my rock, for giving me much needed advice, for being just the opposite to my headstrong soul in unpredictable situations, for giving me higher purpose in my life, for being just my love…

To Elena…

University of Zagreb Doctoral thesis

Faculty of Science

Department of Biology

MORPHOLOGY AND PHYLOGENY OF PICOEUKARYOTES AND PLANKTONIC PENNATE DIATOMS IN THE MIDDLE AND SOUTH ADRIATIC SEA

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The smallest protists, generally called picoeukaryotes, and planktonic pennate diatoms have received limited attention in world oceans, although they are numerous and important for primary production, biogeochemical cycles and for carbon injection into the deep ocean. In this thesis, represented through seven scientific publications, cultivated planktonic pennate diatoms and picoeukaryotes were investigated with traditional methods (light and electron microscopy, morphometry) and with molecular analyses, including multi-gene phylogenies, and were identified as genera *Entomoneis*, *Pseudo-nitzschia*, *Haslea* and *Picochlorum*. Detailed investigations of genus *Entomoneis* resulted with description of seven new species. General picoeukaryotic diversity was addressed by eDNA amplicon sequencing V4 variable region of nuclear 18S rRNA gene. Obtained results identified 95% of the picoeukaryotic community as hetero- or mixotrophic, mostly belonging to parasitic dinoflagellates and radiolarians, while only 5% of the community was represented with photoautotrophic picoeukaryotes belonging to classes of green algae, haptophytes, stramenopiles and cryptophytes. One cultivated *Picochlorum* sp. strain was subjected to growth rate experiment and pigment and lipid analyses, resulting in interesting and promising data for future biotechnological studies.

(52 pages, 3 figures, 232 references, original in English)

Keywords: picoeukaryotes, planktonic pennate diatoms, morphology, phylogeny

Supervisor: Dr Zrinka Ljubešić, Associated Professor

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Dr Regine Jahn, Scientific advisor

Sveučilište u Zagrebu Doktorski rad

Prirodoslovno-matematički fakultet

Biološki odsjek

MORFOLOGIJA I FILOGENIJA PIKOEUKARIOTA I PENATNIH PLANKTONSKIH DIJATOMEJA U SREDNJEM I JUŽNOM JADRANU

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Najmanji protisti, poznati kao pikoeukarioti i penatne planktonske diatomeje često su zanemareni u istraživanjima svjetskih oceana, iako predstavljaju brojnu i važnu komponentu za primarne proizvodnje, biogeokemijskih ciklusa i značajni su za protok ugljika u duboki ocean. U sklopu ove disertacije kroz sedam znanstvenih publikacija navedene skupine organizama istraživane su tradicionalnim metodama (svjetlosna i elektronska mikroskopija, morfometrija) i molekularnim analizama koje uključuju filogenetske anlize sa više različitih genskih markera te su identificirane kao rodovi *Entomoneis*, *Pseudo-nitzschia*, *Haslea* i *Picochlorum*. Detaljna analiza roda *Entomoneis* rezultirala je opisom sedam novih vrsta za znanost. Bioraznolikost ukupne pikoeukariotske zajednice u južnom Jadranu odredila se sekvenciranjem amplikona okolišne DNA i to prema varijabilnoj V4 regiji nuklearnog 18S rRNA gena. Dobiveni rezultati identificirali su 95% pikoeukariotske zajednice kao hetero- ili miksotrofne organizme, većim dijelom pripadajući parazitskim dinoflagelatima i radiolarijama, dok je samo 5% zajednice zastupljeno fotoautotrofnim pikoeukariotima koji pripadaju razredima zelenih algi, haptofita, stramenopila i kriptofita. Jedan kultivirani soj alge *Picochlorum* sp. istražen je kroz eksperiment određivanja brzine rasta te analizama pigmenta i lipida, što je rezultiralo zanimljivim i obećavajućim rezultatima koji mogu poslužiti kao okosnica za buduće biotehnološke studije.

(52 stranice, 3 slike, 232 literaturna navoda, jezik izvornika: engleski)

Ključne riječi: pikoeukarioti, penatne planktonske dijatomeje, morfologija, filogenija

Mentor: Izv. prof. dr. sc. Zrinka Ljubešić

Ocjenjivači: Dr. sc. Daniela Marić Pfannkuchen, znanstveni suradnik

Doc. dr. sc. Sunčica Bosak

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I. **Mejdandžić M**, Bosak S, Orlić S, Gligora Udovič M, Peharec Štefanić P, Špoljarić I, Mršić G, Ljubešić Z (2017) *Entomoneis tenera* sp. nov., a new marine planktonic diatom (Entomoneidaceae, Bacillariophyta) from the Adriatic Sea. Phytotaxa 292: 1-18.

II. **Mejdandžić M**, Bosak S, Nakov T, Ruck E, Orlić S, Gligora Udovič M, Peharec Štefanić P, Špoljarić I, Mršić G, Ljubešić Z (2018) Morphological diversity and phylogeny of the diatom genus *Entomoneis* (Bacillariophyta) in marine plankton: six new species from the Adriatic Sea. Journal of Phycology 54: 275-298.

III. Grbin D, Pfannkuchen M, Babić I, **Mejdandžić M**, Mihanović H, Marić Pfannkuchen D, Godrijan J, Peharec Štefanić P, Olujić G, Ljubešić Z (2017) Multigene phylogeny and morphology of newly isolated strain of *Pseudo-nitzschia mannii* Amato & Montresor (Adriatic Sea). Diatom Research 32: 127-131.

IV. **Mejdandžić M**, Bosak S, Ljubešić Z (2017) Blue Diatoms: Global Phenomenon of ″Greening″ in Shellfish and Record of Planktonic *Haslea* Species in the South Adriatic Sea. Naše more, Znanstveno-stručni časopis za more i pomorstvo 64: 38-44.

V. Ljubešić Z, **Mejdandžić M**, Bošnjak I, Bosak S (2016) Comparing methods in picoplankton abundance estimation. In: Rapp. Comm. int. Mer Médit. 41.

VI. **Mucko M**, Novak T, Medić N, Gašparović B, Peharec Štefanić P, Orlić S, Ljubešić Z (2018) Characterization of newly isolated photosynthetic marine pico green algae (*Picochlorum*, Trebouxiophyceae) from the Adriatic Sea. *Submitted to Acta Botanica Croatica*

VII. **Mucko M**, Bosak S, Casotti R, Balestra C, Ljubešić Z (2018) Picoplankton winter diversity in an oligotrophic marginal sea. *Submitted to Marine Genomics*

THESIS SUMMARY

Free-living microbial populations in pelagic parts of oceans are represented as plankton, diverse collection of organisms passively driven by water currents, which includes organisms belonging to Bacteria, Archaea, Eukarya (protists and fungi) and viruses. The most abundant Bacteria belong to classes *Proteobacteria*, mostly *Alphaproteobacteria* and *Gammaproteobacteria* and phototrophic *Cyanobacteria* (predominantly genera *Prochlorococcus* and *Synechococcus*) while Archeal diversity strongly dominates with classes *Euryarcheota* and *Crenarchaeota*. It is important to emphasize that the newest tree of life published by Hug et al. (2016) shows Bacteria as the most diverse domain with new classes discovered every day, while Archaea and Eukarya, at first sight, in comparison to Bacteria are less diverse. Nevertheless, microbial eukaryotes represent a large range of phototrophic, mixotrophic and heterotrophic organisms with huge morphological and phylogenetic diversity. The most dominant marine microbial eukaryotes belong to super groups Archaeplastida, phyla Chlorophyta and Prasinophyta; Chromalveolata, phyla Dinophyta, Bacillariophyta and MAST (Marine Stramenopiles), classes Pelagophyceae, Cryptophyceae and Prymnesiophyceae; Excavata, phylum Euglenozoa; Opistokontha, phyla Choanoflagellata and Fungi; Rhizaria, phyla Cercozoa and Radiolaria (Massana and Pedrós-Alió 2008). Picoeukaryotes (PEs) are organisms with cell size ≤ 3µm, including phototrophs, heterotrophs, and mixotrophs, which can be involved in mutualistic relationships such as parasitism or symbiosis with larger organisms and are of great importance for biogeochemical cycles in oceans. The diatoms are microscopic eukaryotic algae with unique silicified shell called frustule, and, based on their symmetry and phylogeny can be divided in radial centrics (Coscinodiscophyceae, radial symmetry), bi- or multipolar centrics (Mediophyceae, radial to irregular symmetry), araphid pennates and raphid pennates (Bacillariophyceae, bilateral symmetry), all divided into 9 major phylogenetic clades. In contrast to PEs who have few or non-diagnosable morphological parameters to be identified with, diatoms have a large number of specific morphological features on their frustules and traditionally have been identified primarily with light and electron microscopy. Both PEs and pennate diatoms in marine plankton have often been neglected in microscopical examination of field samples: PEs mostly because of their size, and planktonic pennate diatoms mostly because of their difficult identification using light microscopy, light silification and small abundance. Therefore, the need for the introduction of various molecular methods in their detection and correct identification was acknowledged in last few decades. The diatoms are today commonly identified using nuclear 18S rRNA gene

and chloroplast encoded *rbc*L and *psb*C genes, while PEs, depending on lineages, using nuclear 18S rRNA, plastid 16S rRNA and ITS genes and chloroplast or mitochondrial encoded *rbc*L or COI. However, multi-gene phylogenies are often not possible to obtain since a lot of PEs or pennate diatoms cannot be cultivated. Therefore, next generation sequencing (NGS) methods in examining environmental DNA (eDNA) are introduced in marine phytoplankton research which rely on barcode regions used for species identification and diversity estimations. Additionally, from the biotechnological aspect, some members of pennate diatoms and especially PEs can be interesting and promising.

The aim of this thesis was to obtain a more complete picture of the diversity of marine PEs and pennate planktonic diatoms in the middle and south Adriatic Sea using traditional microscopy, molecular identification and defining physiological attributes, and to try to answer the following important questions during that process: i) Where is the phylogenetical border between species and genus in picoeukaryotes and planktonic pennate diatoms? Does the current knowledge of species/genus border adequately reflect the use and availability of new genetic markers in picoeukaryotes and planktonic pennate diatom research and microscopy in classical morphology? ii) Is the Adriatic Sea a good model for studying shifts in diversity in the plankton communities due to ongoing climate changes? iii) What is the potential for isolation and cultivation of new strains of PEs and planktonic pennate diatoms with the possible biotechnological application?

In this thesis, represented through seven publications, newly isolated and cultivated planktonic pennate diatoms were identified as belonging to the genera *Entomoneis* Ehrenberg, *Pseudo-nitzschia* H. Peragallo and *Haslea* Simonsen while PEs as genus *Picochlorum* W.J.Henley, J.L.Hironaka, L.Guillou, M.A.Buchheim, J.A.Buchheim, M.W.Fawley & K.P.Fawley. Within the genus *Entomoneis*, seven new species were described: *E. tenera*, *E. pusilla*, *E. gracilis*, *E. vilicicii*, *E. infula*, *E. adriatica* and *E. umbratica* (publications **I** and **II**). Further on, Adriatic strain of known species *Pseudo-nitzschia mannii* was characterized (publication **III**), and unknown species *Haslea* sp. and her biotechnologically important pigment marennine was detected (publication **IV**). Additionally, unknown species of *Picochlorum* sp., from genus with yet unknown new species number and great biotechnological potential, was characterized (publications **V** and **VI**). General PEs diversity was addressed by eDNA amplicon sequencing of nuclear 18S rRNA gene, V4 variable region and obtained results identified 95% of the PEs community as hetero- or mixotrophic, while only 5% of the community was represented with photoautotrophic PEs belonging to classes of green algae, haptophytes, stramenopiles and cryptophytes (publication **VII**).

The scientific contribution of this thesis is increasing the general scientific knowledge about the analyzed genera of PEs and diatoms and the descriptions of up to now unknown organisms. Additionally, this thesis provides first NGS analysis regarding PEs in the Adriatic Sea, filling the crucial knowledge gap in plankton studies in this oligotrophic ecosystem. One cultivated *Picochlorum* sp. strain was subjected to growth rate experiments and pigment and lipid analyses, resulting in interesting and promising data for future experimental approaches and biotechnological applications for this highly resilient pico-photoautotroph. Biotechnology in algae, a challenging and promising field of research is with this thesis enriched with green algae strains full of potential for upcoming and evolving varieties of green technologies. With ongoing climate change, studies like this are of the utmost importance for efforts in preserving the ocean and the world’s largest biome.

PROŠIRENI SAŽETAK

Slobodno živuće mikrobne populacije u pelagičkim dijelovima oceana zajednički se nazivaju planktonom - raznovrsnom skupinom organizama pasivno pokretanima vodenim strujama, koji uključuju organizme iz triju domena živog svijeta: Bacteria, Archaea, Eukarya (protisti i gljive), te viruse. Najbrojnije bakterije pripadaju koljenu *Proteobacteria*, najviše razredima *Alphaproteobacteria* i *Gammaproteobacteria* te autotrofnim cijanobakterijama (rodovi *Prochlorococcus* i *Synechococcus*), dok najdominantnije arheje pripadaju razredima *Euryarcheota* i *Crenarchaeota*. Važno je naglasiti da u najnovijem stablu života kojeg su prikazali Hug i suradnici (2016) najveću raznolikost pokazuje upravo domena Bacteria, sa svakodnevno otkrivanim novim razredima, dok je raznolikost arheja i eukariota naizgled puno manja. Usprkos tome, mikrobni eukarioti ipak predstavljaju široki raspon fotoautotrofnih, miksotrofnih i heterotrofnih organizama koji se iznimno morfološki i filogenetski razlikuju. Najbrojniji oceanski mikrobni eukarioti pripadaju supergrupama Archaeplastida, koljenima Chlorophyta i Prasinophyta; Chromalveolata, koljenima Dinophyta, Bacillariophyta, MAST (Marine Stramenopiles), razredima Pelagophyceae, Cryptophyceae i Prymnesiophyceae; Excavata, koljenu Euglenozoa; Opistokontha, koljenima Choanoflagellata i Fungi; Rhizaria, koljenima Cercozoa i Radiolaria (Massana and Pedrós-Alió 2008). Pikoeukarioti su organizmi s veličinom stanica ≤ 3 μm u promjeru, uključuju fotoautotrofe, heterotrofe i miksotrofe koji mogu biti uključeni u mutualističke odnose kao što su parazitizam i simbioza s većim organizmima, a od velike su važnosti za biogeokemijske cikluse u oceanima. Dijatomeje su mikroskopske eukariotske alge s jedinstvenom silificiranom ljušturom zvanom frustula, koje na temelju njihove simetrije i filogenije razlikujemo kao radijalne centrice (Coscinodiscophyceae, radijalna simetrija), bi- ili multipolarne centrice (Mediophyceae, radijalna do nepravilna simetrija) ili arafidne i rafidne penate (Bacillariophyceae, bilateralna simetrija), koje se zajedno dijele unutar 9 filogenetski različitih skupina. Za razliku od pikoeukariota koji ili nemaju uopće, ili imaju samo nekoliko morfoloških parametara, dijatomejske frustule posjeduju velik broj specifičnih morfoloških svojstava te se tradicionalno se identificiraju pomoću svjetlosnog i elektronskog mikroskopa. Pikoeukarioti i penatne planktonske dijatomeje se često predviđaju i zanemaruju u morskim okolišnim uzorcima prilikom klasičnih analiza mikroskopijom: pikoeukarioti uglavnom zbog svoje veličine, a penatne planktonske dijatomeje uglavnom zbog teške identifikacije putem svjetlosne mikroskopije, slabe silificiranosti te male brojnosti u planktonu. Stoga je uvođenje različitih molekularnih metoda u njihovo otkrivanje i ispravnu identifikaciju u posljednjih nekoliko

desetljeća postalo obavezno. Diatomeje se danas obično identificiraju pomoću više različitih gena kao što su nuklearni 18S rRNA gen te kloroplastni *rbc*L i *psb*C geni, dok pikoeukarioti, ovisno o taksonomskim razredima, korištenjem nuklearnog 18S rRNA gena, plastidnog 16S rRNA gena, varijabilne ITS regije te kloroplastnog ili mitohondrijskog gena kao što su *rbc*L ili COI. Ipak, nerijetko identifikacija putem filogenije nije moguća jer se velika većina pikoeukariota i dijatomeja ne mogu uspješno uzgajati u laboratorijskim uvjetima pa se danas uvode i metode sekvenciranja sljedeće generacije (NGS) u različite analize okolišne DNA (eDNA), kako bi se otkrile i identificirale teško uzgojive i rijetke svojte. Osim toga, s biotehnološkog aspekta, neke penatne planktonske dijatomeje, a osobito pikoeukarioti, mogu biti zanimljivi i obećavajući.

Cilj ove disertacije je dobiti potpuniju sliku raznolikosti morskih pikoeukariota i planktonskih penatnih dijatomeja u Jadranu koristeći tradicionalnu mikroskopiju, suvremenu molekularnu identifikaciju i karakterizaciju fizioloških parametara organizama, te u tom procesu pokušati odgovoriti na sljedeća važna pitanja: i) Gdje je granica vrste i roda kod pikoeukariota i penatnih planktonskih dijatomeja? Da li su dosadašnje spoznaje o granicama vrste i roda adekvatne obzirom na korištenje i dostupnost novih genskih markera u istraživanjima pikoeukariota i penatnih planktonskih dijatomeja kao i mikroskopije u klasičnoj morfologiji? ii) Zbog klimatskih promjena dolazi do oligotrofikacije oceana te promjena u planktonskim zajednicama. Da li je Jadransko more idealan model za proučavanje tih promjena zbog svoje oligotrofije? iii) Kolika je mogućnost izolacije novih klonova sa potencijalnom biotehnološkom primjenom?

U ovoj disertaciji, predstavljenoj kroz sedam znanstvenih publikacija, zabilježeni su i istraženi kultivirani dijatomejski rodovi *Entomoneis* Ehrenberg, *Pseudo-nitzschia* H. Peragallo i *Haslea* Simonsen te pikoeukariotski rod *Picochlorum* W.J.Henley, J.L.Hironaka, L.Guillou, M.A.Buchheim, J.A.Buchheim, M.W.Fawley & K.P.Fawley. Rod *Entomoneis* zastupljen je sa sedam novih vrsta: *E. tenera*, *E. pusilla*, *E. gracilis*, *E. vilicicii*, *E. infula*, *E. adriatica* i *E. umbratica* (publikacije **I** i **II**). Nadalje, rod *Pseudo-nitzschia* istražen je s karakterizacijom novog kultiviranog soja poznate vrste *P. mannii* (publikacija **III**), a rod *Haslea* s još nepoznatom 'plavom' dijatomejom *Haslea* sp. čiji je biotehnološki važan pigment marenin zabilježen (publikacija **IV**). Dodatno, rod *Picochlorum* okarakteriziran je sa jednim novoizoliranim sojem velikog biotehnološkog potencijala, dok je broj novih vrsta još uvijek nepoznat (publikacije **V** i **VI**). Opća raznolikost pikoeukariota istražena je sekvenciranjem amplikona eDNA koristeći varijabilnu V4 regiju nuklearnog 18S rRNA gena, a dobiveni su rezultati identificirali 95% zajednice kao heterotrofnu i/ili miksotrofnu, dok je samo 5% zajednice predstavljeno fotoautotrofnim pikoeukariotima koji pripadaju razredima zelenih algi, haptofitima, stramenopilima i kriptofitima (publikacija **VII**).

Znanstveni doprinos ove disertacije je obogaćivanje općeg znanstvenog znanja spomenutih rodova pikoeukariota i penatnih planktonskih dijatomeja s opisima do sada nepoznatih vrsta te karakterizaciji novo izoliranih sojeva poznatih vrsta. Osim toga, ova disertacija daje prvi skup podataka prikupljenih sekvenciranjem sljedeće generacije na Illumina platrofmi koji se odnosi na pikoeukariote u Jadranskome moru, dajući prijeko potrebno znanje planktonskim istraživanjima u ovom oligotrofnom ekosustavu. Biotehnološki potencijal jednog kultiviranog soja roda *Picochlorum* dodatno je analiziran u eksperimentu rasta te analizama pigmenta i lipida, što je rezultiralo zanimljivim i obećavajućim podacima za buduće eksperimentalne pristupe na ovom plastičnom i otpornom piko-fotoautotrofu. Biotehnologija primjenjiva na algama, izazovno i obećavajuće područje istraživanja je s ovom disertacijom dobilo uvid u nove mogućnosti i nove sojeve primjenjive u nadolazećim i rastućim zelenim tehnologijama. Obzirom na prisutne klimatske promjene, studije poput ove su od najveće važnosti za očuvanje oceana, najvećeg svjetskog bioma.

INTRODUCTION

„To many people, 'biodiversity' is almost synonymous with the word 'nature,' and 'nature' brings to mind steamy forests and the big creatures that dwell there. Fair enough. But biodiversity is much more than that, for it encompasses not only the diversity of species, but also the diversity within species.“ - Cary Fowler

The unseen diversity of marine microbial protists

Aquatic ecosystems account for 70% of the Earth's surface (excluding ice and groundwater ecosystems) and preservation of this vast ecosystem is of the utmost importance to humankind (Costanza et al., 1997). Marine ecosystems comprise 97% of aquatic ecosystems in a form of a continuous body of seawater that holds about 320 million cubic miles (1.35 billion cubic kilometres) (NatGeoEd.org). The marine ecosystems are threatened by global changes, such as climate change, overfishing, and pollution, but most of us see just the small picture in form of a decreased number of commercially important fish, marine mammals or increasing amount of plastics and other pollutants in aquatic ecosystems. Most of the world's oceans are unreachable, harboring small amounts of primary producers, consequently called ″blue deserts″, but on the other hand, marine ecosystems harbor a large diversity of microbial populations that ensure their functioning and sustainability.

Free-living microbial populations in pelagic parts of oceans are represented as plankton, diverse collection of organisms passively driven by water currents, which include autotrophic, heterotrophic and mixotrophic organisms: Bacteria, Archaea, members of Eukarya (protists and fungi), and viruses. On average, a liter of seawater contains $\sim 10^6$ eukaryotic cells (Brown et al., 2009), $\sim 10^8$ prokaryotic cells and (Whitman et al., 1998) and $\sim 10^9$-10^{11} virus-like particles (Wilhelm and Matteson, 2008). Plankton organisms dominate marine ecosystem in terms of both abundance and biomass (Zinger et al., 2012), and can be divided depending on their feeding preferences into phytoplankton (plant-like plankton composed of phototrophic protists; cells can perform photosynthesis), zooplankton (animals feeding and grazing on phytoplankton; heterotrophs), bacterioplankton (bacteria, cyanobacteria and archaea) and virioplankton (viruses). Microbial community drives every one of the major biogeochemical cycles that make the ocean processes crucial for all other ecosystems on earth (Worden et al., 2015). Marine microorganisms are a diverse pool of species; for instance, Bacteria within the global ocean are estimated to consist of more than $\sim 2\times 10^6$ taxa (Curtis et al., 2002), Archaea $\sim 2\times 10^4$ taxa (Massana et al., 2000) and 2.2×10^6 Eukarya taxa (Mora et al., 2011).

Protists are diverse eukaryotic organisms mostly found as single cells, although many species form colonies formed of several to numerous cells (Caron et al., 2012). They are distributed throughout all branches of the eukaryotic tree of life (Baldauf, 2008; Burki et al., 2014; **Figure 1**). Together with virioplankton and heterotrophic bacterioplankton, protists form the ″microbial loop″, contributing predominantly to organic matter and nutrient recycling (Azam et al., 1983; Pernthaler, 2005; Pomeroy et al., 2007).

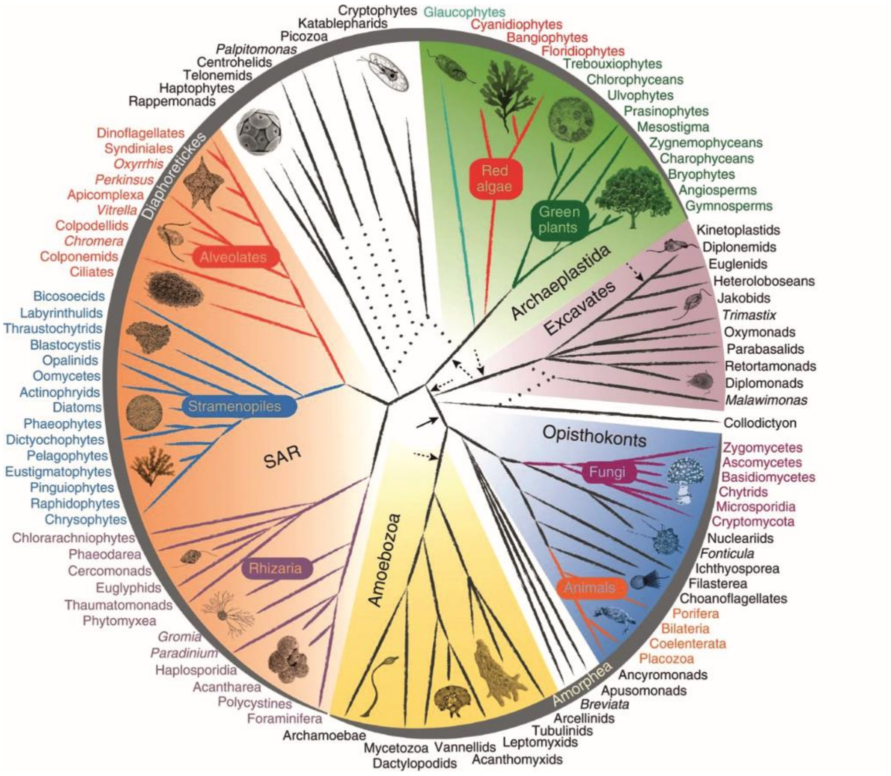

Figure 1. Global tree of eukaryotes from a consensus of phylogenetic evidence (in particular, phylogenomics), rare genomic signatures, and morphological characteristics. Numerous eukaryotic groups are shown (not exhaustively), regardless of their taxonomic rank. Cartoons illustrate the diversity constituting the largest assemblages (colored boxes). The branching pattern does not necessarily represent the inferred relationships between the lineages. Dotted lines denote uncertain relationships, including conflicting positions. Note the solid branch leading to haptophytes and rappemonads. Adapted from Burki et al., 2014.

Major lineages inside photosynthetic protists are Dinophyta (dinoflagellates), Ochrophyta (Stramenopiles, diatoms, golden algae and brown algae), Chlorophyta (Archaeplastida, marine green algae), Haptophyta (mainly coccolithophorids) and Cryptophyta (flagellated microalgae containing phycoerythrin accessory pigments) (Anderson et al., 2011; Wang et al., 2016; Tragin et al., 2017). Generally, protists can be divided into size fractions: pico- (0.2 to 2(3) µm), nano- (2(3) to 20 µm) and micro- (20 to 200 µm) fraction (Sieburth et al. 1978). Smallest protists, generally called picoeukaryotes (PEs), and pennate planktonic diatoms (size fractions nano and micro; 3-200 µm) received limited attention in world oceans, although they are numerous and important for both primary production, as well as for carbon injection into deep ocean (Worden et al., 2004; Agusti et al., 2015). PEs have especially important roles in oligotrophic ecosystems, where together with picocyanobacteria (such as *Prochlorococcus* and *Synechococcus*) are the driving force of primary production, but they are even more important at high latitudes, where marine cyanobacteria are less numerous (Lovejoy et al., 2007; Balzano et al., 2012; Flombaum et al., 2013).

Marine protists can be investigated with traditional techniques such as light, electron and epifluorescence microscopy, pigment analysis with High-Performance Liquid Chromatography (HPLC), flow cytometry, etc. Additionally, other tools can be applied in protist studies, depending on the area of research (abundance, phylogenetic diversity, cultures and functional diversity, **Figure 2**). Morphology, as a study of external and internal structure, shape and form of organisms with exhaustive observations of various details specific to each taxon, and phylogeny as a study of evolutionary relationships between organisms are the most used ways of investigating living organisms, among them protists.

Fundamentally, there is widespread acceptance that identification of protist species using light microscopy alone is no longer sufficient or adequate. Light microscopy has very limited use to assess picoplankton diversity in the field, but in cultures it can provide valuable information on their size, shape of the cells, number of plastids, swimming or sliding behavior, consequently allowing identification of the species. Additionally, specific fixatives, such as Lugol or osmium can make certain cellular features (flagella) more visible or the fixatives can destroy the samples. Descriptions based on microscopy and holotypes deposited in designated institutes as fixed slide preparations or photographs can help to describe a morphotype but fail to identify species (Adl et al. 2007). More improved electron microscopy (transmission or scanning electron microscopy) allows to describe to a certain extent in morphological diversity of larger protist cells (for example dinoflagellates or diatoms), but it is limited when applied to small protists (Moestrup and Throndsen, 1988). Electron microscopy can allow us to determine specific ornamentation of cells, as was shown for minute *Bathycoccus prasinos* W. Eikrem & J. Throndsen or *Imantonia rotunda* N. Reynolds (Eikrem and Throndsen, 1990). However, usage of electron microscopy has some limitations. Cells need to be fixed and concentrated before embedding or mounting, which can result in the loss of small or delicate forms. Microscopes are usually too big to carry to field and demand specific environment (certain temperature and light conditions) to be adequately placed. Epifluorescence microscopy relies on the emission of light by cellular compounds (e.g. pigments) or by strains specific for certain components such as DNA. The vast diversity of heterotrophic protists is difficult to examine under light and electron microscopy, so staining and epifluorescence microscopy has proven to be a better tool in investigations of these cells (Sherr and Sherr, 1993).

Pigmented protists (i.e. phytoplankton) possesses a collection of pigments which are somewhat specific to certain lineages, such as classes (for example fucoxanthin and diatoxanthin in diatoms, peridinin and diadinoxanthin in dinoflagellates or prasinoxanthin in prasinophytes) (Wright and Jeffrey, 2006). Pigment analysis expanded rapidly in the 1980s with the development of automated HPLC methods for pigment separations, useful for routine shipboard or shore-based determinations (e.g. Wright and Shearer, 1984; Roy, 1987). HPLC method allows separation of up to 40 pigments - chlorophylls, carotenoids and their derivatives. Types of microalgae that had previously been missed in microscopic analysis of field samples through filtering loss or preservation damage could now be recognized from their pigment signatures (e.g. Gieskes and Kraay, 1983; Guillard et al., 1985). However, HPLC as a method has some disadvantages: (i) due to the liability and rapid degradation of phytoplankton pigments, special conditions must be employed to preserve samples, and samples must contain certain concentration of pigments so they can be detected (ii) these pigments are particularly sensitive to light, heat, oxygen, acids and alkalis; (iii) whilst a number of pigments is specific to particular classes or genera, a number of other pigments is spread across many algal classes making interpretation of the data difficult (iv) expression of the pigments is quite variable with the contents of a particular cell varying with a number of environmental factors such as irradiance and nutrients. Despite these disadvantages, HPLC is still the best technique for mapping phytoplankton populations and monitoring their abundance and composition.

Pigmented cells can be quantified using flow cytometry, a technique able to separate cells based on their size and auto-fluorescence (Marie et al., 1997). Flow cytometry is an analytical technique, based on optical properties and/or specific fluorescence of particles (cells) and their constant flow through the flow chamber/counting chamber within a sheat fluid. Flow-cytometry measures and enumerates small cells and particles such as phytoplankton, bacteria, and viruses. Light scattered by each individual cell (a function of cell size and refractive index) and fluorescence from pigments such as chlorophyll or phycoerythrin are recorded in real time (Marie, 1999). Major advantages of flow cytometry include speed, accuracy, and absence of sample preparation, at least to analyze photosynthetic pigments. This revolutionary method led to the discovery of very important picoplanktonic organisms such as *Prochlorococcus* (Chisholm, 1988) and *Ostreococcus* C. Courties & M.-J. Chrétiennot-Dinet (Courties et al., 1994). Still, scattering and fluorescence properties are not sufficient to discriminate taxa within picoeukaryotes, with the exception of cryptophytes that contain phycoerythrin (Li and Dickie, 2001). Therefore, potential applications of flow cytometry are considerably enhanced when samples are stained with fluorescent markers binding to specific cell compounds.

The tiny proportion of cultivable marine protists, our inability to identify them, interpret their diversity and to study them in detail all contribute to huge knowledge gaps, which are often difficultly bridged (Massana, 2011; del Campo et al., 2014).

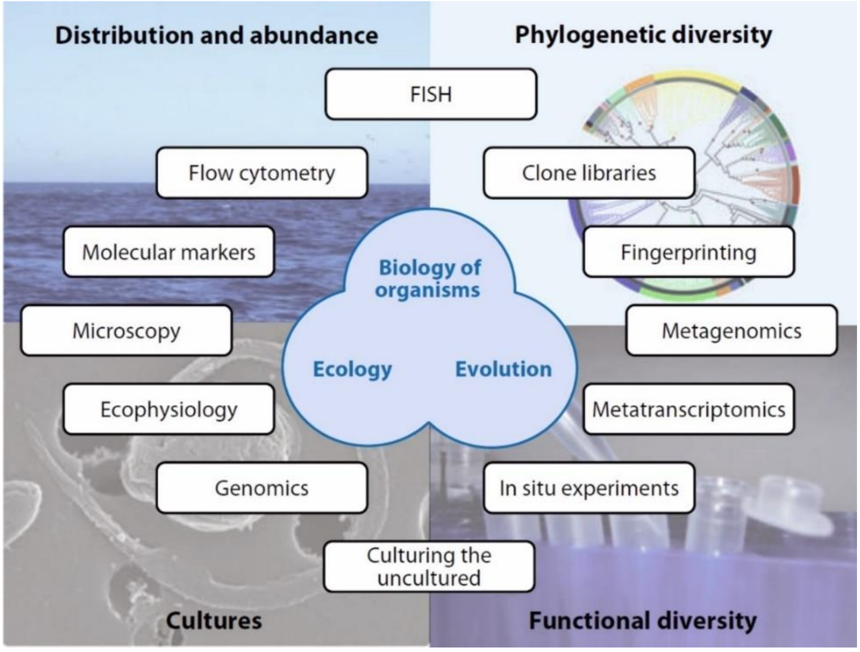

Figure 2. Overview of approaches to investigate cell biology, ecology, and evolution of marine protists, treating four main study areas: distribution and abundance, phylogenetic diversity, functional diversity and culture studies. Abbreviation: FISH, fluorescence in situ hybridization. Adapted from Massana, 2011.

One universal method to access diversity among all organisms is applicable to genes and proteins which present a certain degree of variability (Anne, 2006). Molecular markers can be divided into three categories (Schlötterer, 2004): the protein variants (i.e. allozymes), the DNA (Deoxyribo Nucleic Acid) sequence variations (i.e. polymorphisms) and the DNA repeat variation. Remarkable progress in molecular biology and science altogether brought revolutionary methods such as the Sanger method (Sanger and Coulson, 1975) and Polymerase chain reaction (PCR; Saiki et al., 1985). Later on, next-generation sequencing (NGS) or high-throughput sequencing (HTS) methods such as 454 pyrosequencing (based on Ronaghi et al., 1998) in 2005 (Margulies et al., 2005), and then Illumina sequencing (based on Canard and Sarfati, 1994) in 2007. HTS methods allowed the transition between clone libraries sequenced by Sanger method and the large metabarcoding datasets, where Sanger sequencing provides a relatively low number of long high-quality sequences, while HTS provides a large amount of medium-quality sequences and allows only small fragments to be sequenced. Several different gene markers to assess the protist diversity have been used in HTS methods: V4 region of 18S rRNA gene (Massana et al., 2014; Zimmermann et al. 2011); V9 region of 18S rRNA gene (De Vargas et al., 2015); *rbc*L (large subunit of the ribulose-1,5-biphosphate carboxylase-oxygenase) encoded in plastid genomes; and cox1 (cytochrome c oxidase subunit I) gene encoded in mitochondrial genomes (Kermarrec et al., 2013); 16S rRNA plastid gene (Lepère et al., 2009; Choi et al., 2017); or more species specific hypervariable region such as ITS (internally transcribed spacer of the rRNA operon) (Coleman, 2003; Rodríguez-Martínez et al., 2013). These sophisticated sequencing methods, called metabarcoding led us to ambitious projects such as Tara Oceans (https://oceans.taraexpeditions.org/en/m/about-tara/les-expeditions/tara-oceans/) or Ocean Sampling Day project (https://www.microb3.eu/osd.html), which together obtained large and useful datasets of marine eukaryotic diversity. Along with metabarcoding, metagenomics can allow us to investigate community gene repertoires and metabolic potential, whereas metatranscriptomics can provide insights into realized functions (Dinsdale et al., 2008; Frias-Lopez et al., 2008).

Picoeukaryotes: significance and diversity

Picoeukaryotes (PEs) are single-celled ubiquitous organisms that possess a nucleus and a minimal number of organelles (mitochondrion, Golgi apparatus, optionally flagellum, and chloroplasts if photosynthetic; then called photosynthetic picoeukaryotes (PPEs)), and like the rest of eukaryotes, their cellular components are the evolutionary products of endosymbiosis which occurred once or several times in their evolution (Delwiche, 1999). These organisms are the smallest organisms among eukaryotes, having cells between 0.2 to 3µm in diameter, including phototrophs, heterotrophs, and mixotrophs, which can be involved in mutualistic relationships with larger organisms such as parasitism or symbiosis (Acosta et al., 2013). PEs are an important constituent of the ocean's microbiota and perform essential roles in biogeochemical cycles, and form, together with prokaryotes, an ocean’s veil above which larger protists and metazoans might bloom (Massana, 2011).

PPEs account for a significant fraction of primary production, especially in oligotrophic conditions (Li, 1995; Worden and Not, 2008). Although numerically less abundant than marine cyanobacteria, PPEs constitute a third active group of the marine picophytoplankton which was reported several times: in the North Atlantic (Li et al., 1992); Arabian Sea (Shalapyonok et al., 2001); equatorial Pacific (Mackey et al., 2002); Sargasso Sea and Mediterranean Sea (DuRand et al., 2001; Brunet et al., 2007). PPEs reach 1-3×10^3 cells mL^{-1} in oligotrophic systems and up to 10^5 cells mL^{-1} in nutrient rich coastal zones (Sanders et al., 2000; Li, 2009). Together with cyanobacteria *Prochlorococcus* and *Synechococcus* they form the picophytplankton, which accounts for a high fraction (80-90%) of phytoplankton biomass in the oceans (Latasa and Bidigare, 1998; Not et al., 2008). They can be extremely abundant in some areas, such as California, USA, coastal site where PPEs were responsible for up to 76% of the net picoplanktonic production (Worden et al., 2004). Most dominant PPEs in world oceans proved to be Prymnesiophyceae, Prasinophyceae (Mamiellophyceae), Cryptophyceae, Pelagophyceae, Chrysophyceae, and Dictyochophyceae (Shi et al., 2009). Their role in the food chain can be of most importance, as some studies showed that PPEs are subjected to grazing more than cyanobacteria (for example *Synechococcus*), profoundly because of their size (cells larger than coccoid cyanobacteria) which can be substantial for carbon transfer to higher trophic levels (Stockner, 1988). This has direct consequences in packaging carbon in larger particles, which contributes to biological carbon pump whereby organic carbon is transferred to the deep ocean.

Heterotrophic PEs (HPEs) are generally considered bacterial grazers (Jürgens et al., 2008). They keep bacterial stocks stable, transfer dissolved organic matter to higher trophic levels and recycle nutrients that sustain regenerated primary production (Massana, 2011). Although considered less abundant than PPEs, HPEs proved to be very abundant in the oceans, defined by high operational taxonomic unit (OTU) numbers (De Vargas et al., 2015). Many studies showed great prevalence of HPEs over PPEs in oligotrophic ocean ecosystems, where was previously considered that PPEs are of an extreme importance for primary production (Shi et al., 2009, Acosta et al., 2013, De Vargas et al., 2015, Pernice et al., 2015, Pearman et al., 2017). Interactions between HPEs and prokaryotes have ecological implications, as bacterial abundances and community composition are strongly influenced by the predation pressure of HPEs (Jardillier et al., 2005). Besides primary production and bacterivory, HPEs can also influence different trophic levels through parasitic and mutualistic symbiotic associations (Worden and Not, 2008).

General PE diversity is still unexplored, due to various causes: i) small eukaryotic cells cannot be easily identified with traditional methods; ii) many are not easily cultivated in artificial media and laboratory conditions; iii) due to their uneven cellular properties, some taxonomic groups preserve better than others (Vaulot et al., 2008). Most of the cultured PPEs belong to Prasinophyceae, Pelagophyceae, Bolidophyceae, and Pinguiphyceae, while HPEs to

Bicosoecida and Chrysophyceae (Vaulot et al., 2008; Jürgens et al., 2008). Most of picoeukaryotic taxonomic groups do not possess easily discriminated characteristics visible under light microscopy at the current resolution levels (Johnson and Sieburth, 1982; Andersen et al., 1996). With the development of electron microscopy, such as TEM, important diagnostic features were better detected (presence and shape of flagellar hairs or body scales, the presence of pyrenoids and starch inclusions, chloroplast organization and membrane configuration (Eikrem and Edvardsen, 1999)). Culturing through enrichment cultures, pre-filtered cultures, flow-cytometry sorted or manually isolated and serially diluted techniques allowed scientists to describe and culture various picoplankton species (e.g. *Micromonas pusilla* (Butcher) Manton & Parke (Butcher, 1952)) that in the end drastically improved our world collections of PPEs (Vaulot et al., 2004; Andersen and Kawachi, 2005). At the simplest level, photosynthetic pigments (as a key taxonomic diagnostic feature for microalgae) allows us to distinguish green, brown and red algae, but HPLC signature is often indicative of the class (e.g. prasinoxanthin is only present in Prasinophyceae) (Guillou et al., 1999). Due to inherent limitations of the above methods, for decades PEs were treated as a ″black box″ of difficult access (Massana, 2011). Molecular tools gave new insights into the microbial world and revolutionized microbial ecology (Massana, 2011). As mentioned in the previous section ″*The unseen diversity of marine microbial protists*″, HTS methods greatly improved general knowledge on PEs diversity in last decade, allowing us to identify uncultivable representatives and rare taxa.

Diatoms: significance and diversity

Diatoms (Bacillariophyta) are unicellular, mostly photoautotrophic heterokonts (a group of algae with golden-brown to brown chloroplasts originating from a red algal endosymbiont), often forming colonies, with cell sizes roughly between 10 and 200 µm. Some diatoms can live heterotrophically in the dark if supplied with a suitable source of organic carbon, while less than ten species are obligatory heterotrophs (from the genera *Nitzschia* Hassall and *Hantzschia* Grunow) (Round et al., 1990). Their unique hallmark is the ornamented compound silica cell wall called a frustule built of an amorphus hydrated silica ($SiO_2 \times nH_2O$) and organic material (proteins, polysaccharides). The frustule consists of two halves, unequal in size: smaller hypotheca and larger epitheca, each containing its valve – hypovalve and epivalve, which are held in place by silicified girdle bands called hypocingulum and epicingulum. The typical valve looks like a Petri dish with a flat area called the valve face and a rim called mantle (both cingulum together called mantellum). The flux of material across the frustule takes place via multiple sorts of pores or slits, while the cytoplasm is fully protected (Round et al., 1990). With their almost indestructible shells, conveniently small size, great variety and beauty, diatoms were perfectly ‘pre-adapted’ to their role as the objects of scientific fashion. There are a significant number of morphologic characters to be considered on each diatom frustule, most of them being in common for all diatoms (e.g. areolae, striae, virgae, raphe, fultoportulae or rimoportulae, central and apical pores, girdle bands areolae, etc.); but a lot of characters are genus, and/or species-specific, and need to be carefully investigated when observing a diatom frustule and identifying a species or discriminating against two similar species. Diatoms’ photosynthesis apparatus holds primary chlorophylls (Chl *a* and *c*) and protective xanthophyll (fucoxanthin, diadinoxanthin, diatoxanthin) and carotene (β-carotene).

Historically, diatoms were divided into two major groups: the centrics and the pennates, which can be distinguished by their symmetry (centrics having radial symmetry, while pennates having bilateral symmetry), mode of sexual reproduction (oogamous centrics, while isogamous pennates; **Figure 3**) and plastid number and structure (discoid plastids in centrics, while plate-like plastids in pennates) (Round et al., 1990). Morphologically, pennates can be further divided into two groups by the presence or absence of a slit (raphe) in the valve for movement, i.e. motile pennates possessing raphes are ‘raphid’, whereas immotile pennates lacking raphes are ‘araphid’ diatoms. Today, the most accepted classification system presents diatoms in three classes: Coscinodiscophyceae (radial centric diatoms), Mediophyceae (bi- or multi- polar centric and some radial centric diatoms) and Bacillariophyceae (pennate diatoms) (Medlin and Kaczmarska, 2004). However, newest phylogenetical classification resolves evolution of diatoms as radial centrics grading into polar centrics, which grade into araphid pennates, which themselves grade into the monophyletic raphid pennates (Theriot et al. 2015). According to the phylogenetical division, diatoms are encompassed in 9 major phylogenetic clades: **R1** (*Leptocylindrus*, *Tenuicylindrus*, *Corethron*), **R2** (*Ellerbeckia*, *Proboscia*, *Melosira*, *Aulacoseira*, *Paralia*, *Endictya*, *Stephanopyxis*, *Podosira*), **R3** (*Rhizosolenia*, *Guinardia*, *Coscinodiscus*, *Actinocyclus*, *Actinoptychus*, *Aulacodiscus*), **P1** (*Thalassiosira*. *Cyclotella*, *Triceatirum*, *Odontella*, *Biddulphia*, *Attheya*, *Lithodesmium*, *Ditylum*, *Eunotogramma*), **P2** (*Ceratulina*, *Eucampia*, *Hemiaulus*, *Chaetoceros*, *Bacteriastrum*, *Acanthoceros*, *Urosolenia*), **P3** (*Trigonium*, *Lampriscus*, *Stictocyclus*, *Isthmia*, *Climacosphenia*, *Chrysanthemodiscus*, *Toxarium*, *Ardissonea*), **A1** (*Striatella*, *Asterionellopsis*. *Bleakeleya*, *Delphineis*, *Rhaphoneis*, *Plagiogramma*), **A2** (*Fragilaria*, *Synedra*, *Staurosira*, *Licmophora*, *Diatoma*, *Opephora*, *Tabularia*, *Asterionella*, *Grammatophora*, *Thalassionema*), **Raphid pennates** (*Eunotia*, *Nitzschia*, *Pseudo-nitzschia*, *Fragilariopsis*, etc.) (Theriot et al. 2015). Genera *Entomoneis*, *Pseudo-nitzschia* and *Haslea*, which are investigated in this thesis belong to the monophyletic raphid pennate diatoms, the youngest evolved diatoms in general (Theriot et al. 2015). The genus *Entomoneis* belongs to the specific canal-raphe diatoms from the lineage Surirellales (Ruck and Theriot 2011), while genus *Pseudo-nitzschia* which is sister to *Fragilariopsis*, anchored into the paraphyletic genera *Nitzschia* and *Haslea*, which are sisters to diverse genus *Navicula*, belonging to the canal-raphe lineage Bacillariales (Li et al. 2017; Ruck and Theriot 2011). From the evolutionary perspective, *Pseudo-nitzschia* evolved first, *Haslea* followed, and *Entomoneis* evolved most recently (Ruck and Theriot 2011). *Pseudo-nitzschia* and *Haslea* have a common ancestor in their canal raphe evolution, while taxa from the genus *Entomoneis* have a different ancestor, which is responsible for the evolution of its raphe canal on elevated keel, common for the orders Surirellales and Rhopalodiales (Ruck and Theriot 2011, Ruck et al. 2016).

Diatom reproduction is particularly interesting, usually by mitosis, but with the reduction in cell sizes (**Figure 3**). When diatom cell undergoes mitosis, each daughter cell receives one of the two halves of the frustule, leaving daughter cell to synthesize its own hypotheca. After sub-sequential mitosis, diatoms diminish, leading to the critical point for sexual reproduction. In centrics, this process is oogamous, while in pennates is isogamous and includes ″+″ and ″–″ cells (as ″female″ and ″male″ cells) which need to get close together to exchange gametes. A zygote then buds into auxospore that expands to the initial vegetative diatom cell size.

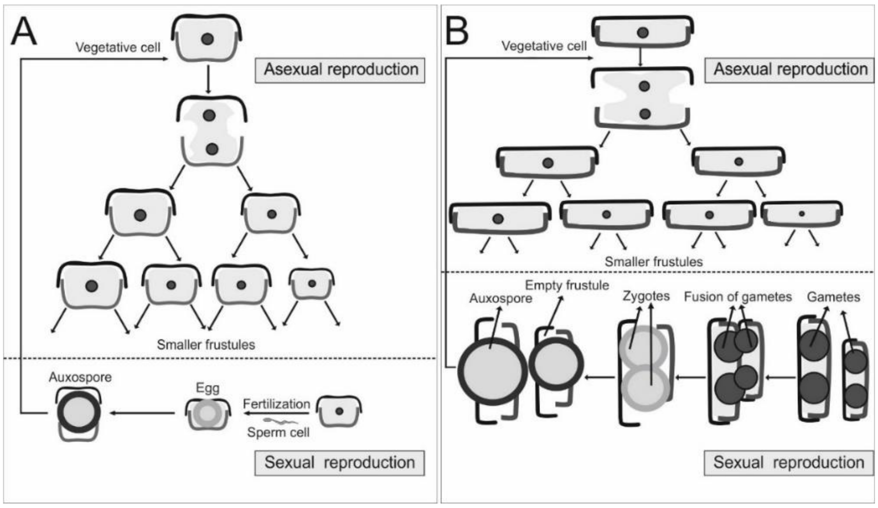

Figure 3. Reproduction in diatoms. A: Oogamous centrics; B: Isogamous pennates

Diatoms commonly live in various terrestrial and aquatic habitats with enough water (or moisture), nutrients, sun energy and CO_2 allowing them to perform photosynthesis and to create simple sugar molecules (glucose) and O_2 as a by-product. Generally, diatoms live either a planktonic or benthic life, where in the plankton are found most commonly radial and bi- or multipolar centrics and a small amount of pennates, whereas pennates predominantly inhabit benthos (on various types of substrates: rocks, plant material, sediment, animals, etc.). Many diatoms secrete kinds of polysaccharides which encapsulate the cells, so they can use it to create all sorts of pads or stalks to attach to various surfaces, or to form colonies. Additionally, to endure in extreme environmental conditions, many diatom species can form resting stages, either in forms of auxospores or dormant vegetative cells (Round et al., 1990). Planktonic diatoms are the most successful group in phytoplankton, obtaining more than 20% of world's carbon dioxide fixation, which in total exceeds carbon uptake by rain forests. Furthermore, they contribute approximately between 20% (Mann, 1999) and 45% (Yool and Tyrrell, 2003) of the global net primary production. Diatoms are abundant in nutrient-rich coastal ecosystems and at high latitudes and their diversity is currently estimated to ca. 200,000 species (Mann and Droop, 1996), but it is still debated among phycologists. Some estimations made by Guiry (2012) gave a conservative figure of 12,000 described species of diatoms and 8,000 yet to be discovered. However, Mann and Vanormelingen (2013) estimated at least 30,000 but possibly up to 100,000 species. Regardless of which of these estimates are more accurate, there is no doubt that large fraction of diatom diversity is yet to be described or even collected.

Generally, marine planktonic centric diatoms received greater scientific attention historically and today, taking into account their more noticeable morphology and abundance in light microscopy, while tender planktonic pennate diatoms (although they can have large cells) are often neglected in field samples. Additionally, centrics (both radial and multipolar) had been in the scientific focus because of their adaptations to the planktonic lifestyle in form of various colony formation (chains, common in genera *Chaetoceros* Ehrenberg, *Bacteriastrum* Shadbolt, *Thalasiossira* Cleve, *Skeletonema* Greville) and girdle elements expansion (e.g. *Rhizosolenia* Brightwell, *Proboscia* B.G. Sundstrom). Shape and length of chains in centric diatoms seem to be a compromise between various environmental factors, such as the effectiveness of grazer defense, adjustment of buoyancy or optimization for nutrient uptake (Reynolds, 1984; Karp-Boss et al., 1996). A small number of pennate planktonic diatoms adapted to planktonic lifestyle also form colonies, such as the genera *Asterionellopsis* Round, *Asteroplanus* C. Gardner & R.M. Crawford in R.M. Crawford & C. Gardner (helical winding colonies), *Thalassionema* Grunow ex Mereschkowsky (zigzag or stellate colonies), *Thallasiothrix* Cleve & Grunow (radiate colonies), *Pseudo-nitzschia* (needle-like chain colonies) and *Fragilariopsis* Hustedt (ribbon-like colonies) (Kooistra et al., 2007). The last two genera have been investigated the most among planktonic pennate diatoms, but even so, this amount of investigations is still scarce in comparison to centric diatoms (Kang and Fryxell, 1992; Lundholm et al., 2003; Trainer et al., 2012; Lim et al., 2018; Aslam et al., 2018). A possible reason why so few pennate diatoms are found in the plankton may have to do with their isogamous sexual reproduction, where isogametes are amoeboid and need to crawl to one another, or because single-celled pennates have been neglected in plankton due to their low numbers, light silification, small size and morphological characters only visible under electron microscopy. Nevertheless, pennates in plankton deserve more investigations globally.

Characterization of diatom diversity requires accurate and consistent taxon identification. Where morphological analyses alone often fail to provide a complete description, complementary approaches are performed to provide a uniform means of standardization in estimations of global diatom diversity (Logares et al., 2014). With the introduction of HTS methods in diatom research, biodiversity studies such as Tara Oceans, Malaspina and others, are beginning to bridge the knowledge gaps by generating large amounts of genetic and phenotypic data and uncovering biodiversity patterns at global scales (Nealson and Venter, 2007; De Vargas et al., 2015; Agusti et al., 2015). These studies have discovered novel diversity around species and genera which historically have been considered relatively species poor (e.g., *Planktoniella* F. Schütt; Malviya et al., 2016) and suggest that there are likely many other groups of planktonic diatoms that are similarly understudied. Most abundant species in marine plankton according to HTS results of big surveys belong to the genus *Chaetoceros*, followed by *Fragilariopsis*, *Thalassiosira* and *Corethron* Castracane (Malviya et al., 2016).

Species concepts in protistology

Nonexistence of a generally accepted concept for delimiting protists species has many unfortunate consequences (Boenigk et al., 2011). The first is a lack of basic communicability about fundamental biological units, with obvious negative implications for barcoding; the second is a lack of clarity regarding their evolutionary and ecological significance, while third is a drastic underestimation of protist diversity and importance in more general biodiversity papers (Boenigk et al., 2011). Looking back on the beginnings of taxonomical investigations of protists, scientists were faced with limitations of light, then scanning and transmission electron microscopy, but molecular methods at the beginning of the 1990’s have brought new light into protistology, allowing many new lineages to be discovered. A recent approach for overcoming the drawbacks of insufficient taxonomic coverage of the studied diversity is the use of operational taxonomic units (OTUs) (Green et al., 2004). In many cases, OTUs are treated synonymous to species and are used for estimating species richness (Boenigk et al., 2011). Nevertheless, we are often faced with the inconsistencies between molecular phylogenies on the one hand and morphological species denominations and traditional classification concepts on the other. Due to different methodology in species identification, protist diversity can often be misinterpreted. For instance, the existence of cryptic species (two or more genetically distinct species classified as a single species due to morphological identity) can result in increased biodiversity estimations; or by contrast, taxon-independent (OTU-based) diversity studies (often applied to microorganisms) can result in underestimation of natural biodiversity (Boenigk et al., 2011). Some of the key issues in alpha diversity among protist are: i) confusion arising from high levels of evolutionary convergence and morphological conservation; ii) morphological plasticity which can lead to unreliable species diagnoses; iii) different rates of phenotypic and genotypic divergence, meaning that a single genetic marker will provide inconsistent taxonomic signal; iv) uncertainty about diagnosable morphological characters for defining a genus/species; v) high levels of molecular diversity that are not correlated with known cells (from natural or cultivated material) leading to much higher genotypic than morphological diversity; vi) limited genetic information about many protist lineages (Boenigk et al., 2011).

Today, there are ca. 24 existing species concepts that can be considered, which is futile and ignores the biological realities (Boenigk et al., 2011). Among them, the most often discussed and applied concepts are the biological species concept (BSC), morphological species concept (MSC), phylogenetical species concept (PSC) and ecological species concept (ESC).

The BSC is a complex set of different processes important for delimiting species. In the essence, BSC defines a biological species as groups of interbreeding natural populations that are reproductively isolated from other such groups. The emphasis of this definition is not on the degree of morphological differences, but genetic ones. For example, some scientist agree that each species should be isolated reproductively, while others agree to allow a certain percent of species hybridization (with at least one other species, not necessarily being sister species) (Mallet, 2008). Additionally, we certainly cannot rule out horizontal gene transfer as a ″invasion″ into a genome, a problem in species delimitation when a single gene marker is used (but more on that in the section about PSC). When adapted to PEs, BSC considers that two populations belong to the same species if they can interbreed and their descendants are fertile. This can be particularly difficult to assess as most of PEs are asexual clonal organisms, with short periods of specific sexual reproduction, happening either when conditions in the environment are unfavorable, or in particular seasons (Grimsley et al., 2009 and references therein). Likewise, the BSC when applied to diatoms, where it is also based on separation of species according to their inability to interbreed, this concept is also problematic due to mostly unknown sexual reproduction (Medlin, 2018). Three genera, which are well studied regarding sexual reproduction (*Pseudo-nitzschia*, *Melosira* Agardh, and *Haslea*), represent indeed just a tip of an iceberg in the diatom world (Mouget et al., 2005; Mizuno, 2006, 2008; Kaczmarska and Ehrman, 2015). The examples are *Haslea karadagensis*, *H. provincialis*, *H. ostrearia* and *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima* (Mouget et al. 2005; Gastineau et al. 2012; Gastineau et al. 2016). It is particularly complicated to interbreed *Pseudo-nitzschia* or *Haslea* species in cultures, as both ″+″ and ″-″ cells are needed to reproduce, normally present in dioecious species (Davidovich and Bates 1998). In *Pseudo-nitzschia*, allogamy is common mode of sexual reproduction where gamete production starts 2-3 days after mixing cells from different clonal cultures. Two types of sexes (″+″ and ″-″) cross and exchange gametes (2 per cell). Rearrangement of gametangia is not synchronous and may start first in either the larger or the smaller cell. Afterwards, plasmogamy happens and two large axospores form. Finally, two initial cells form within the auxospore (Davidovich and Bates 1998). In *Haslea*, sexual reproduction begins with two gametes per cell and then syngamy occurs, resulting in zygotes. Zygotes then grow and form tubular auxospores. Initial vegetative cells (one per auxospore) form within the auxospores (Gastineau et al. 2016).

The MSC relies on unique and discernible morphological characters that can be observed in a cell which can lead to species identification. However, in marine PEs, most of the unknown diversity is a direct consequence of the impossibility to get a pure culture, which is often necessary to define a species. Observations with light and electron microscopy can provide discernible morphological characters only in a small number of PEs, therefore, the MSC is in most cases inapplicable to the smallest eukaryotes (Potter et al., 1997; Rosselló-Mora and Amann, 2001). In diatoms, probably the most used and the applicable concept is morphological because of their intricate cell walls (Williams and Kociolek, 2007; Cox, 2009). However, this concept is not ‘foolproof’ because of diminishing cell size in cell division and morphological changes from the initial cell to the smallest one (more pronounced in pennate diatoms, subtler in centrics). Additionally, some diatom species can express different morphologies when living under different conditions (seasons or habitats) (Klee and Houk, 1996; Rose and Cox, 2014; Wetzel et al., 2015).

As phenotypic (morphological) characters of species, discernible by light or electron microscopy are difficult to distinguish or are highly plastic within a species, it is important to combine phenotypic with genotypic data to delineate species, mostly using several gene markers (Boenigk et al., 2011). This is a base of the PSC, which states that separate species or higher taxonomical levels must be monophyletic. Monophyletic groupings are clusters of individuals that are diagnosable distinctly from other clusters and should display a parental pattern of ancestry and descent, independent of the marker used (Cracraft, 1989; Vanderlaan et al., 2013). In marine PEs, PSC can also be difficult to assess, since many PEs cannot be cultivated and are analyzed with different molecular markers. Therefore, during the last decade, PSC is sometimes transformed into a concept based on OTUs (Pedrós-Alió, 2006). PEs are more and more treated as prokaryotes in estimations of diversity, making the definition of a species less essential. Many studies investigating PEs diversity use OTUs as ‘species’ and delineate them on criteria of minimal similarity thresholds and number of clones (more clones, more accurate taxonomical delineation) representing an OTU (Pedrós-Alió, 2005). In diatoms, PSC is more thoroughly investigated, and it was estimated that all diatom species are monophyletic. Additionally, thanks to fossil records, we can apply molecular clocks to divergences and get an estimation of species separation and evolution (Medlin, 2018; Souffreau et al., 2011). Species can be estimated in the phylogenetic concept through cladistics analysis of their frustule components (Kooistra et al., 2010; Edgar et al., 2015; Pennesi et al., 2016); through geometric morphometric analyses (Beszteri et al., 2005a; Edgar et al., 2015) or through sequence analysis of one or more genes (Theriot et al., 2015). Problems arise when some authors accept paraphyletic groupings (emergence of a new taxa from different parent taxa, splitting the parent taxa into minimally two, or more new taxa; e.g. *Pinnularia borealis* Ehrenbeg complex, Pinseel et al., 2017), and others do not, resulting in loss of well known taxa (e.g. *Hemidiscus* Wallich, Gómez et al., 2017). Therefore, diatomists often cannot emphasize enough how important it is to combine molecular data with other species concepts (morphological, biological or ecological) to estimate a ‘species’. As a powerful tool, molecular data can identify multi-species complexes (cryptic species) and help to define species limits (Medlin, 2018). Additionally, molecular data can provide an objective framework to be used in determining physiological and morphological differences in taxonomic identification, and can also interpret gene flow and dispersal mechanisms, allowing us to better understand the biogeography of a certain species (Medlin, 2018).

Finally, the ESC also provides useful information about strain occupation of certain niche, where two strains that occupy different niches are considered to belong to different species (Dykhuizen, 1998). On the other hand, the ecological species concept is very complicated to define, as an ecological niche of an organism relies on several physical-chemical and biological parameters that need to be considered simultaneously.

Combination of mentioned species concepts is popular, probably mostly the phylo-phenetic species definition, more applicable in prokaryotes, but also used for small eukaryotes, relies on information from 18S rRNA gene. It is accepted that two strains must show a similarity >97% to be considered part of the same species (Rosselló-Mora and Amann, 2001); but on the other hand, the same authors highlighted that 18S rRNA gene does not have enough resolution to delineate species. Such a rigid definition of species in marine PEs certainly cannot be easily accepted, so we must take into consideration both concepts – the morphological and phylogenetical. Nevertheless, every species concept has its benefits and drawbacks, leaving scientists to use each of them with caution and apply them as best they can to their organisms (Pedrós-Alió, 2006).

Adriatic Sea: dynamic and oligotrophic ecosystem and a natural laboratory

The Adriatic Sea, situated as the northernmost part of the Mediterranean Sea, with the length of 800 km extending from the Strait of Otranto to the northwest Po River valley, the width of 200-250 km and an average depth of 252 m, is a semi-enclosed oligotrophic basin bathymetrically divided into three areas. In the north, the Adriatic Sea is shallow (maximum depth 50 m), the middle Adriatic basin is deeper with a depth up to 280 m, while the southern part is characterized by a deep Southern Adriatic Pit (SAP) (maximum depth 1234 m). This partition also identifies distinct physical (Artegiani et al., 1997) and biological (Zavatarelli et al., 1998) oceanographic characteristics. The average temperature of the Adriatic Sea ranges between 22 and 24 °C in summer and from 12 to 14 °C in winter, while the salinity generally varies between 38 and 39 (Cushman-Roisin et al., 2013).

The Adriatic Sea is defined with two main currents – East Adriatic Current (EAC) and Western Adriatic Current (WAC). EAC brings highly saline and low-nutrient waters from Ionian and Levantine Seas, while WAC carries large amounts of high-nutrient freshwater from the Po River, which makes the Adriatic Sea a quite heterogeneous marine system with across-shelf and longitudinal trophic gradients (Polimene et al., 2006). Contributing to the dense water formation for the eastern Mediterranean deep circulation cell, southern Adriatic presents the entering point for water masses originating from the Ionian Sea: Ionian surface water (ISW) and Levantine intermediate water (LIW, Zore-Armanda, 1963) (Polimene et al., 2006). Dense water generation sites are located in the Adriatic Sea; the dense water sinks to the deep Ionian Sea and the Levantine Basin (Gačić et al., 2010). In the North Adriatic Sea, dense water is formed (the North Adriatic Dense Water (NAdDW), Zore-Armanda, 1963), which is generated during severe and cold bora wind outbreaks (Beg Paklar et al., 2001; Mihanović et al., 2013). Further, in the middle Adriatic Sea the stationary water mass (the Middle Adriatic Deep Water (MAdDW), Zore-Armanda, 1963) with low oxygen concentrations resides when no strong NAdDW generation takes place. Finally, the Adriatic Deep Water (AdDW, Zore-Armanda, 1963) is formed in the South Adriatic Sea which is generated in the SAP during cold bora outbreaks through deep-convection processes (Gačić et al., 2002). AdDW is spreading to the deep Ionian Sea and influences the vorticity balance there, therefore shrinking the water column along the western perimeter, changes the geostrophic balance between the perimeter and the inner Ionian, and induces anticyclonic circulation (Vilibić et al., 2012). The anticyclonic circulation then drags a branch of the modified Atlantic water (MAW) towards the Adriatic, which is characterized by lower salinity, temperature, and density, resulting in the generating of the NAdDW and AdDW of lower density (Vilibić et al., 2012). NAdDW and AdDW then flow towards the north-western Ionian Sea where together with less dense MAW lying above them, stretch the water column and change geostrophic balance, resulting in a shift of the circulation to the cyclonic one, and this important concept is called Bimodal Adriatic-Ionian Oscillation (BiOS) (Gačić et al., 2010). This concept explains a number of known phenomena such as decadal salinity oscillations in the Adriatic called the Adriatic ingressions (Buljan, 1953; Civitarese et al., 2010) and the decadal variation of the MAW meandering in the Ionian Sea (Malanotte-Rizzoli et al., 1997). Importance of these physical concepts is in direct consequence to the biogeochemical properties and biodiversity of the whole Eastern Mediterranean Basin, where the primary production is low and limited by phosphorus (Siokou-Frangou et al., 2010). The only exception is the northernmost Adriatic, where high river nutrient loads are responsible for the phytoplankton blooms and eutrophication of this area (Degobbis and Gilmartin, 1990; Degobbis et al., 2000). Additionally, the circulation may be quite important on interannual and decadal scales over the specific areas of the Mediterranean Basin like the Adriatic Sea. Phenomena like horizontal or vertical advection and displacement of nutrients may cause shifts in primary production at specific locations, such as the deep convection locations, e.g. the SAP where phytoplankton blooms follow the deep convection events (Gačić et al., 2002; Vilibić and Šantić, 2008). The Adriatic Sea upper layer circulation is driven by the freshwater input, particularly of the northern Adriatic rivers that result in a cyclonic surface circulation, with the outflowing WAC along with the western coastline and the inflowing LIW and the surface Ionian waters along the eastern coastline. The impact of nutrient load in northern Adriatic is mainly restricted to the same area and the WAC (Grilli et al., 2005; Polimene et al., 2006; Solidoro et al., 2009), as the nutrients are normally consumed very fast during their transport towards the southeast and the open Adriatic (Campanelli et al., 2011). By contrast, the open Adriatic Sea nutrient load is mostly controlled by the inflowing waters coming from the Ionian Sea (Šolić et al., 2008). Therefore, the inflow of the intermediate waters from the Ionian Sea, mainly of the LIW, is a major supplier of the open Adriatic nutrients, and it is found to influence the Adriatic long-term productivity (Marasović et al., 1995, 2005; Grbec et al., 2009). Civitarese et al. (2010) applied the BiOS concept to the observed biological changes in the Adriatic and found a correlation between high salinity periods and allochthonous organisms coming from the Eastern Mediterranean. Furthermore, they found correlation between allochthonous organisms coming from the Western Mediterranean and low salinity periods.

The two coasts of the Adriatic Sea also differ greatly, the west one being alluvial or terraced, while the eastern is highly indented with pronounced karstification. This karstic environment developed from the Adriatic Carbonate Platform in the Oligocene and Miocene when the mountain chain Dinarides uplifted (Surić et al., 2005). Along the western coast with strong discharge from Po River, higher nutrient content can give rise to higher dissolved and particulate organic carbon production to extreme ecological phenomena such as dystrophic events and, consequently, anoxia in the bottom layers of the water column (Polimene et al., 2006). The middle and southern regions of the Adriatic Sea are characterized by lower primary production and extreme oligotrophy with the continental input and the benthic pelagic interactions being of minor importance in comparison to the northern area (Zavatarelli et al., 2000). Nevertheless, there are some isolated coastal micro-areas which can show a certain degree of eutrophication, mostly due to anthropogenic influence, but generally, the middle and southern Adriatic Sea is extremely oligotrophic. Likewise, the Adriatic Sea is small, certainly when compared to oceans, and has shorter time of response to shifts in physico-chemical conditions. These are the reasons that the Adriatic Sea can be generally considered a natural laboratory for many biological questions, as well as physical or chemical ones (Vilibić et al. 2017).

THESIS OUTLINE

This thesis summarizes seven scientific publications (**I – VII**) in which all the aims and questions of the thesis are addressed.

Aims of this thesis are: 1) Detailed morphological and phylogenetical analyses of cultivated PEs and pennate planktonic diatoms; 2) An exhaustive molecular analysis of eDNA in order to show the general diversity of marine PEs in the Adriatic Sea; 3) Description of potentially new species of planktonic pennate diatoms.

Questions of this thesis are: 1) Where is the phylogenetical border between species and genus in picoeukaryotes and planktonic pennate diatoms? Does the current knowledge of species/genus border adequately reflect the use and availability of new gene markers in picoeukaryotes and planktonic pennate diatom research and microscopy in classical morphology?; 2) Is the Adriatic Sea a good model for studying shifts in diversity in the plankton communities due to ongoing climate changes?; 3) What is the possibility of isolating new strains with potential in biotechnology?

Publications **I** and **II** directly contribute to first and third aim, as well as first question, with seven newly described species of diatom genus *Entomoneis,* isolated in middle and southern Adriatic Sea, which were examined morphologically using light and electron microscopy and phylogenetically using three gene markers (nuclear 18S rRNA and plastid *rbc*L and *psb*C). Publication **III** contributes to first aim and first question. This is accomplished by the characterization of the newly isolated strain from the middle Adriatic Sea of the known species *Pseudo-nitzschia mannii* with light and transmission electron microscopy and three-gene phylogeny using nuclear 18S rRNA, 28S rRNA and ITS variable region. Publication **IV,** on the other hand, contributes to the third question with the characterization of newly isolated strain of a yet undescribed species of the genus *Haslea* with light microscopy, discussing the importance of blue-pigmented diatoms and biotechnological potential of the pigment marennine. Publications **V** and **VI** contribute to the first aim and first and third questions, characterizing one strain of pico-green trebouxiophyte, *Picochlorum* sp. isolated from the South Adriatic Sea and describing its biotechnological potential through growth rate, pigment and lipid experiments and analyses. Publication **VII** contributes to the second aim where PEs are analysed with amplicon sequencing of variable V4 region of nuclear 18S rRNA gene through Illumina platform and general diversity is defined for the first time in the Adriatic Sea. The remaining, second thesis question, is discussed combining all publications.

INDIVIDUAL PUBLICATIONS

„Taxonomy is described sometimes as a science and sometimes as an art, but really it’s a battleground.“- Bill Bryson

Publication I

Phytotaxa 292 (1): 001–018
http://www.mapress.com/j/pt/
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Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.292.1.1

Entomoneis tenera sp. nov., a new marine planktonic diatom (Entomoneidaceae, Bacillariophyta) from the Adriatic Sea

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Abstract

The genus *Entomoneis* Ehrenberg includes diatoms with structurally complex frustules having a bilobate keel elevated above the valve surface and numerous girdle bands. We describe here a new member of the genus, *Entomoneis tenera sp. nov.*, a minute species found in the plankton of the south-eastern Adriatic Sea. The description and proposed taxonomic affiliation are based on both morphological observations and molecular analyses obtained from three cultivated strains. The cultures were established from plankton net samples taken during BIOTA (Bio-tracing Adriatic Water Masses) cruise conducted in February–March 2015. In addition to characteristic *Entomoneis* features such as panduriform cells, often twisted around the apical axis and a raphe with simple endings positioned on the sigmoid keel, morphological characteristics of *E. tenera* are: (1) very small cells, 16–21 µm long and 5–20 µm wide (2) very lightly silicified, delicate frustules without valve striation discernible in light microscopy, (3) broad lanceolate valves with scalpeliform apices (4) a straight to slightly arcuate junction line. Phylogenetic analyses using SSU, *rbc*L and *psb*C supported the position of *E. tenera* within the *Entomoneis* genus with a clear separation from the other described species.

Key words: marine diatoms, new species, *Entomoneis*, phytoplankton, Adriatic Sea

INTRODUCTION

Genus *Entomoneis* Ehrenberg (1845: 71, 154) comprises mostly epipelic species commonly observed in brackish to marine sediments, occasionally found in freshwater (Round *et al.* 1990). *Entomoneis* taxa are mostly found in low abundances with very few records of their occurrence in large numbers in plankton (McMinn & Hodgson 1993) or benthos (Dalu *et al.* 2015).

The history of the genus starts with Ehrenberg who described a new species *Navicula alata* Ehrenberg (1840: 212) and a new genus *Amphiprora* (1843: 401) with *Amphiprora constricta* (1843: 25) as a type species. Several years later he reinterpreted his *Navicula alata* as *Entomoneis alata* (Ehrenberg) Ehrenberg (1845: 154) and created a new genus named *Entomoneis.* Patrick & Reimer (1975) established the nomenclatural priority of *Entomoneis* as a valid name for all species with parapically oriented sigmoid elevation (winged keel), panduriform shaped frustule, numerous intercalary bands, juncture of the keel with the valve body in form of a junction line, raphe raised on a sigmoid keel and bi- or multiseriate striae.

Additionally, Patrick & Reimer (1975) established the Entomoneidaceae family which included *Entomoneis* and *Plagiotropis* Pfitzer (1871: 189), but the latter genus has been subsequently transferred to the family Plagiotropidaceae D.G. Mann (Round *et al.* 1990). Currently, Entomoneidaceae includes two genera: *Entomoneis* and recently described *Platichthys* (Lange-Bertalot, Kulikovskiy, Witkowski, Seddon & Kociolek, 2015), having common morphological characteristics such as raphe canal and fibulae, compressed valve face, absence of distinct valve mantle and numerous copulae. However, unlike species belonging to *Entomoneis*, the cells of *Platichthys* species do not have twisted panduriform frustule, they possess only monoseriatae striae and they do not have sigmoid keel (Lange-Bertalot *et al.* 2015).

Accepted by Christopher S. Lobban: 22 Dec. 2016; published: 20 Jan. 2017

From taxonomical, ecological and evolutionary point of views, the genus *Entomoneis* together with 30 other genera (*e.g. Nitzschia* Hassall 1845, *Tryblionella* W. Smith (1835: 35), *Psammodictyon* D.G. Mann in Round *et al*. (1990: 675), *Pterodictyon* D.G. Mann in Round *et al*. (1990: 674), *Giffenia* Round & Basson (1997: 348), *Archibaldia* Witkowski & Kociolek in Witkowski *et al*. (2011: 172), *Platichthys*, *etc*.) belong to the canal-raphe-bearing diatoms, a non-monophyletic group where the raphe canal evolved independently on two separate occasions (Ruck & Theriot 2011). According to Ruck & Theriot (2011) and Ruck *et al.* (2016), phylogenetic analyses using chloroplast and three-gene datasets (SSU, *rbc*L and *psb*C) strongly supported monophyletic origin of the genus *Entomoneis*. Although morphologically similar to *Entomoneis* the phylogenetic position of *Platichthys* is unknown, therefore the monophyletic origin of the family Entomoneidaceae remains to be confirmed.

Of the 45 *Entomoneis* species and intraspecific taxa names currently included in the Algaebase, only 21 are considered taxonomically valid (Guiry & Guiry 2016). In the late 1980's and early 1990's several studies examined brackish/marine samples and described new taxa such as *Entomoneis alata* var. *japonica* (Osada & Kobayasi 1985) later brought to species level as *E. japonica* (Cleve) K. Osada in S. Mayama, M. Idei, K. Osada & T. Nagumo (2002: 89), *E. decussata* (Grunow) K. Osada & H. Kobayasi (1990: 260), *E. aequabilis* (Osada & Kobayasi 1991), *E. centrospinosa* (Osada & Kobayasi 1990b), *E. punctulata* (Grunow) K. Osada & H. Kobayasi (1990: 163) and *E. pseudoduplex* K. Osada & H. Kobayasi (1990: 165) each accompanied with detailed morphological characterization. From the recent descriptions of new species (e.g., *E. oestrupii* (Van Heurck) Cremer (2003: 102), *E. reimeri* D.C. Reinke & Wujek (2013: 116), *E. vertebralis* (Clavero *et al*. 1999), one fossil diatom emerged as well, *E. calixasini* C. Paillès, M.M. Blanc-Valleron & M. Poulin in Paillès *et al*. (2014: 413) which was described from Turkish Marmara Sea sediments. In the Adriatic Sea, several *Entomoneis* species are recorded: *E. alata* occurs both in plankton and benthos, while *E. paludosa, E. paludosa* var. *duplex* (Donkin) Makarova & Akhmetova (1987: 53), *E. pulchra*, and *E. ornata* occur only in benthos (Viličić *et al.* 2002; Cibic & Facca 2010).

Overall, in the past two decades, only four new *Entomoneis* species have been described exclusively on the basis of their morphology. In this study, *Entomoneis tenera sp. nov.,* a very small and weakly silicified taxon found in the marine plankton in the Adriatic Sea is described as a new species. The description is based on both morphological and molecular information obtained from the cultured strains. The similarities and differences with other species within the genus are also discussed.

MATERIAL & METHODS

Sampling and cultures

During the BIOTA (Bio-tracing Adriatic Water Masses) cruise conducted in February–March 2015 in the south-eastern Adriatic Sea, the seawater samples containing *Entomoneis tenera sp. nov.* were collected with 20µm-pore-size mesh plankton net at two stations: P600 (N 42°24′ E 17°55′) and M300 (N 42°29′ E 17°17′). Monoclonal cultures of three strains: PMFEN1, PMFEN2 and PMFEN3 were established by micropipette isolation using an Olympus CKX41 inverted light microscope (Olympus, Tokyo, Japan). The strains were kept in culture flasks filled with 30 mL of f/2 medium (Guillard's f/2 Marine Water Enrichment Solution, Sigma-Aldrich, United Kingdom). The cultures were maintained under cool-white (40-W) fluorescent light (30 µmol photons m^{-2} s^{-1}) at room temperature (18°C–19°C) with a 16:8 light:dark cycle and sub-cultured every week.

Morphological analysis

Light microscopy observations were performed with an inverted Zeiss Axio Observer Z1 (Carl Zeiss, Oberkochen, Germany) microscope equipped with DIC and phase contrast and Olympus BX51 (Olympus, Tokyo, Japan) microscope. The removal of organic matter from diatom frustules followed Simonsen (1974) and Hasle (1978). The samples (5 mL) were first rinsed with distilled water, followed by the addition of 5 mL of saturated $KMnO_4$ and left for 24 hours. The next day 5 mL of 36% HCl was added, and gently heated over an alcohol burner flame until it became clear or only slightly coloured and then rinsed at least five times with distilled water until free of acid. Permanent slides were prepared by drying cleaned material on cover slips and mounting in Naphrax (Brunel Microscopes Ltd.), following Hasle (1978). All three cultivated strains were examined with the same morphological and molecular approach. PMFEN2 was chosen as a referent strain for holotype material and permanent slide stored at Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany under accession number BRM ZU10/75.

For scanning electron microscopy (SEM), culture material was directly filtered on 3µm-pore-size Nucleopore polycarbonate filter (Nucleopore, Pleasanton, CA). Dehydration was done with the ethanol-series (25, 35, 50, 75, 80, 90%) prepared with distilled water and absolute ethanol, finishing with three rinses of 100% ethanol. Hexamethyldisilazane (HMDS) treatment was used for drying the samples (Bray *et al.* 1993). The sample was rinsed in a series of 100% ethanol: HMDS solutions (3:1, 1:1, 1:3), finishing with three rinses of 100% HMDS, 5 min treatment at each step, allowing the last HMDS rinse to evaporate slowly at a room temperature. The filters were placed on aluminium stubs, coated with 15 nm gold using Scancoat Six Sputter Coater (BOC Edwards, Wilmington, Mass., U.S.A.) and examined with FEG Tescan MIRA3 microscope (Brno, Czech Republic). For transmission electron microscopy (TEM), cleaned culture material was deposited onto Formvar-coated grids, air dried and examined with FEI Morgagni 268D microscope (Eindhoven, The Netherlands).

The general diatom terminology used for the morphological descriptions follows Ross *et al.* (1979). More specific terminology for *Entomoneis* follows Paddock & Sims (1981) and Osada & Kobayasi (1985).

Molecular analysis

Genomic DNA was isolated from 50 mL of cell cultures obtained in exponential phase of growth. Cultured cells were collected centrifuging for 15 min at 2000×g using SL 16R centrifuge (Thermo Fisher Scientific©, Waltham, USA). The pellet was re-suspended in Eppendorf tube with 0.5 mm glass beads (BioSpec Products Inc., Fisher Scientific, Waltham, MA, USA) and vortexed for 10 min. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen©, Heiden, Germany) according to manufacturer's instructions. The purity of the extracted DNA was assessed with the NanoDrop spectrophotometer (BioSpec-nano, Shimadzu, Kyoto, Japan). One nuclear (SSU rDNA or 18S rDNA) and two chloroplast (*rbc*L, *psb*C) DNA regions were amplified using the Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, California, USA) following the PCR protocol as described in Ruck & Theriot (2011). Nested PCR reactions were done for obtaining *psb*C genes of all three strains and SSU rDNA of PMFEN3 using a PCR product from first reaction as a template for second reaction. The primers used for amplification are listed in Table 1. PCR products were visualized in a 1% agarose gel and then purified with StartaPrep PCR Purification Kit (Agilent Technologies, Santa Clara, California, USA). The purified products were sequenced by Sanger dideoxy sequencing method (Macrogen, Amsterdam, The Netherlands).

TABLE 1. Primers used to amplify SSU rDNA, *rbc*L and *psb*C fragments in this study. Primers in bold were used for nested PCR reaction.

Primer name	Primer sequence (5'–3')	Reference
SSU1	AAC CTG GTT GAT CCT GCC AGT	Medlin *et al*. 1988
ITS1DR	CCT TGT TAC GAC TTC ACC TTC C	Edgar & Theriot 2004
SSU11+	TGA TCC TGC CAG TAG TCA TAC GCT	Alverson *et al*. 2007
SSU1672-	TAG GTG CGA CGG GCG GTG T	Ruck & Theriot 2011
*rbc*L66+	TTA AGG AGA AAT AAA TGT CTC AAT CTG	Alverson *et al*. 2007
dp7-	AAA SHD CCT TGT GTW AGT YTC	Daugbjerg & Andersen 1997
*psb*C+	ACA GGM TTY GCT TGG TGG AGT GG	Alverson *et al*. 2007
*psb*C-	CAC GAC CWG AAT GCC ACC AAT G	Alverson *et al*. 2007
***psb*C22+**	CGT GGT GAT ACA TAG TTA	Ruck & Theriot 2011
***psb*C1154-**	GCD CAY GCT GGY TTA ATG G	Ruck & Theriot 2011

Phylogenetic analysis

Terminal regions of each gene were manually trimmed using BioEdit Sequence Alignment Editor 7.2.5 software (Hall, 1999) to minimize the percentage of missing data. All sequences were checked and paired (5'–3'and 3'–5' ends) using Sequencher ver. 4.1.4. (Gene Codes, Ann Arbor, MI, USA). BLAST analysis was done for all sequences with blastn tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Alignments (both SSU rDNA and chloroplast (CPL) gene phylogenies) were done using AliView with default parameters and checked by eyeball. Accession numbers of newly generated sequences are listed in Table 2, and all other sequences used in the alignments are available in supplement (see Electronic Supplement with Table S1). Following Ruck & Theriot (2011), five datasets were analysed: (1) the nuclear SSU rDNA (see Electronic Supplement with Fig. S1); (2) chloroplast *psb*C (see Electronic Supplement with Fig.S2); (3) chloroplast *rbc*L (see Electronic Supplement with Fig. S3); (4) concatenated alignment including two chloroplast genes–*rbc*L and *psb*C (CPL dataset) and (5) concatenated alignment with all three genes: SSU rDNA, *rbc*L and *psb*C. SSU rDNA phylogeny and concatenated SSU rDNA, *rbc*L and *psb*C phylogeny was done on the sequences belonging to a single clone (PMFEN3), due to unsuccessful SSU rDNA sequences amplification of clones PMFEN1 and PMFEN2. Maximum likelihood (ML) analysis were tested using MEGA 6 software (Tamura *et al*. 2013) with GTR+G+I model as the best suited model according to Best DNA modeltest on all the alignments. Each analysis included 1000 bootstrap replicates. Additionally, bayesian inference (BI) analyses were performed using MrBayes v3.1.2. (Huelsenbeck & Ronquist 2001) on all five datasets, each using default priors and the GTR+G+I model. Posterior probabilities were assessed in two runs using four MCMC chains with trees and parameters sampled every 1000 generations. Number of generations and burn-in information for each dataset is available in Table 3. Stationarity was confirmed using Tracer ver. 1.5. (Rambaut & Drummond 2007). All trees were visualised with FigTree v1.4.3. (available at http://tree.bio.ed.ac.uk). Afterward, the consensus phylogenetic trees were made for each dataset.

TABLE 2. Sequence accession numbers related to the sequences deposited in the GenBank database regarding amplified gene and location of sampling.

Strain	Gene	Sampling location	Accession number
PMFEN1	*psb*C	M300 (N 42°29′ E 17°17′)	KX591884
PMFEN1	*rbc*L	M300 (N 42°29′ E 17°17′)	KX591885
PMFEN2	*psb*C	M300 (N 42°29′ E 17°17′)	KX591886
PMFEN2	*rbc*L	M300 (N 42°29′ E 17°17′)	KX591887
PMFEN3	*psb*C	P600 (N 42°24′ E 17°55′)	KX591888
PMFEN3	*rbc*L	P600 (N 42°24′ E 17°55′)	KX591889
PMFEN3	SSU	P600 (N 42°24′ E 17°55′)	KX591890

TABLE 3. Sequence data, evolutionary models and Log-likelihood values (-ln L) from ML estimations.

Parameter	SSU	*rbc*L	*psb*C	Combined CPL	Combined 3-gene
Number of sequences	37	35	26	26	24
Final aligned length (bp)	1513	1366	1075	2551	4230
Bayesian runs (used)	2(2)	2(2)	2(2)	2(2)	2(2)
Bayesian generations	15M	15M	15M	40M	60M
Bayesian burn-in	1.5M	1.5M	1.5M	4M	6M
ML/BI model (AIC)	GTR+G+I	GTR+G+I	GTR+G+I	GTR+G+I	GTR+G+I
MLE -ln L	6140.389	7888.67	6545.77	14159.45	21601.565

RESULTS

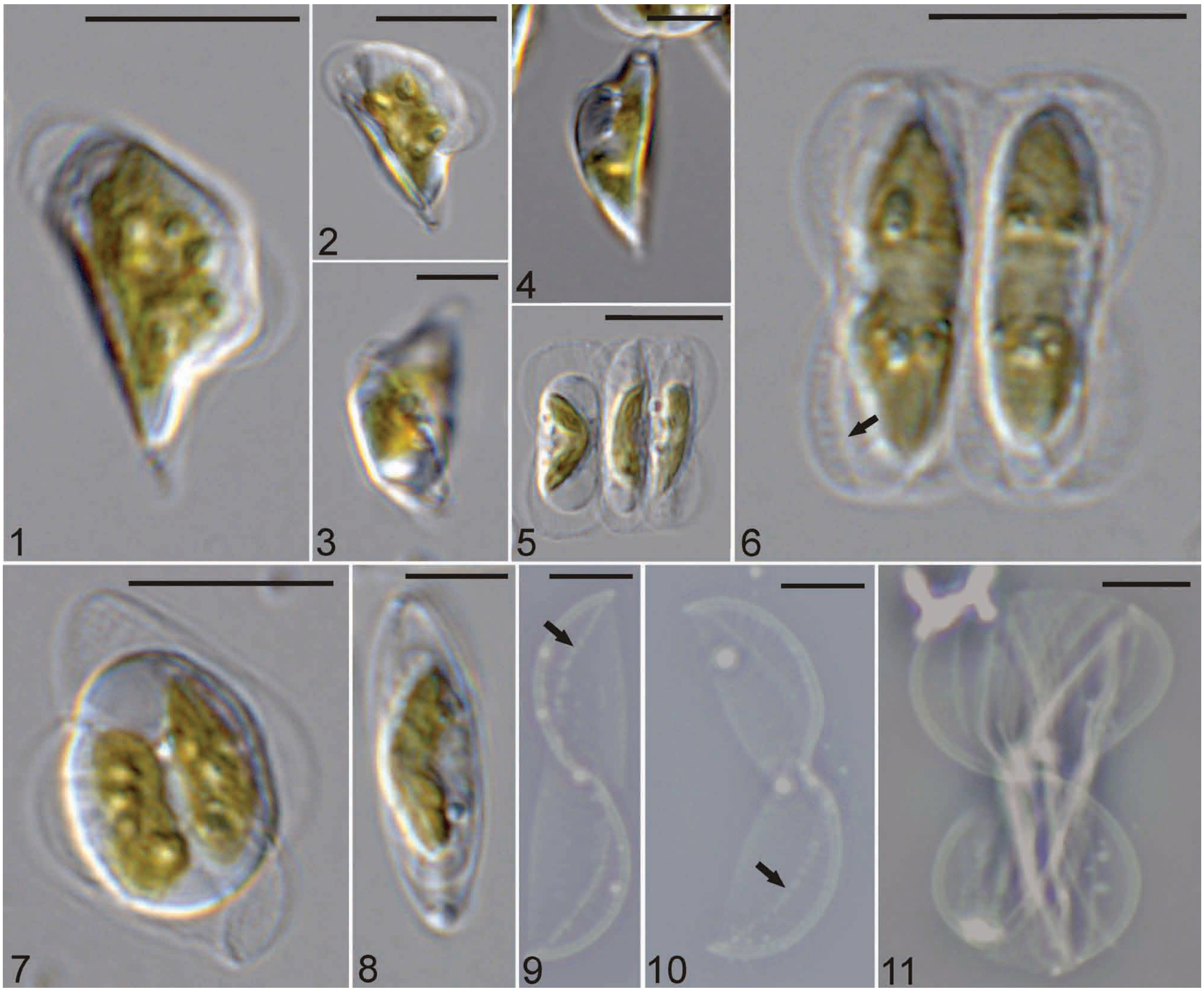
Division **Bacillariophyta**

Class **Bacillariophyceae** Haeckel 1878 emend. D. G. Mann in Round *et al*. 1990

Order **Surirellales** D. G. Mann in Round *et al*. 1990

Family **Entomoneidaceae** Reimer in Patrick & Reimer 1975

Genus ***Entomoneis*** Ehrenberg 1845



FIGURES 1–11. *Entomoneis tenera*, LM. Live cells (Figs 1–8); cleaned material (Figs 9–11). Figs 9–11 taken from holotype permanent slide BRM ZU10/75. (1–4) Cells with various degree of torsion along the apical axis. (5, 6) Recently divided cells. Arrow in Fig. 6. shows the junction line. (8) Lanceolate valve. (9) Valve with sigmoid keel and scalpeliform apices. (10) Girdle view of the valve with straight to arcuate junction line (arrows in Figs 9, 10). (7, 11) Panduriform cell. Scale bars: Figs 8, 1, 2, 5, 6, 7=10 µm; Figs 3, 4, 9, 10, 11=5 µm.

Entomoneis tenera Mejdandžić & Bosak *sp. nov.* (*Figs* 1–35)

LM morphology: Cells delicate with very lightly silicified frustules. One multi-lobbed chloroplast. Frustules panduriform in girdle view, constricted in half of the frustule length, often twisted around the apical axis with the different degrees of torsion (Figs 1–7). Cells 16–21 µm long, 5–15 µm wide in constricted central part and 7–20 µm in widest part. Valves broad lanceolate (Fig. 8), 11–22 µm long and 3–7 µm wide in central part. Scalpeliform valve apices (Figs 9, 10). Sigmoid raphe-bearing keel distinct in valve view (Fig. 9). Elevated keel separated from the valve body with straight to slightly arcuate junction line (Figs 6, 9). In girdle view, junction lines are positioned at an angle of about 45° from the apical axis (Fig. 10). Junction lines are sometimes hardly visible and valve striation is undiscernible in LM due to the light silification of frustules (Fig. 11).

EM morphology: Valve striation becomes apparent in EM (Figs 12–15). The transapical costae and striae are arranged parallel on the valve body, extending from the valve margin towards the junction line (Figs 14–20). Costae are straight and simple, continuous from valve margin to keel, sometimes bifurcated at the valve margin or near the junction between valve body and wing (Figs 14–17). Valve striae 30–55 per 10 µm. Strongly bilobate wing elevated from the valve body with wing costae and striae following contour of the keel, fusing along the junction line and further continuing parallel near the raphe to give a radial appearance (Figs 14–16). Wing striae 18–42 per 10 µm. The striae are closed by a hymen with rectangular perforations arranged in two parallel lines along the stria edges (Figs 19–22). Arrangement and density of the perforations similar in striae on both valve body and wing, 20–39 per 1 µm near the valve margin and 26–37 per 1 µm near keel margin (Figs 19–21). Series of basal fibulae born on each wing costa form a junction line

(Figs 16, 18, 21, 22); 60–70 basal fibulae per 10 µm. Basal fibulae sometimes interconnected with adjacent fibulae with transverse connections in shape of H or W (Fig. 21). Sigmoid raphe with simple linear central and terminal endings (Figs 23–28). The raphe slit is plicate, located at the apex of the keel. The raphe canal is separated from the valve by raphe fibulae, except in the central nodule which is three to four costae wide (Figs 23, 24). Raphe fibulae 29–42 per 10 µm. The central and terminal raphe endings are simple and very slightly curved downwards at valve apices (Figs 25–28). The cingulum is composed of one valvocopula and three to four copulae with smooth external surface (Figs 29–30) and similar ultrastructure with two rows of distinct, elongated areolae (Figs 31–33). In several observed valvocopulae, teardrop shaped areolae, with more elongated drop apex and larger radius in abvalvar than in advalvar ones (Fig. 31). Between each two abvalvar teardrop shaped areolae, silica thickenings (Fig. 31). Areola density in valvocopulae 40–50 per 10 µm. Oblong areolae in copulae are occluded by very lightly silicified hymenes perforated with round to rectangular poroids (Figs 34, 35).

Type:—CROATIA: Adriatic Sea, south-eastern coast (N 42°29’ E 17°17’). Plankton net sample collected on March 2, 2015 by S. Bosak. Holotype slide of the strain PMFEN2 deposited in The Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany as BRM ZU10/75 (holotype illustrated in Figs 9, 10, 11). PMFEN1 and PMFEN3 permanent slides deposited as isotypes at Macedonian diatom collection, Skopje, Macedonia under accession numbers MKNDC /Acc. No. 10517, MKNDC/ Acc. No. 10518, respectively.

Etymology:—From Latin adjective *tenera* (soft, delicate, gentle, fragile) referring to the delicate valves and general appearance of the cells in light microscope.

Habitat:—Marine plankton.

Comments:—Summarized morphological features of *E. tenera* and comparison with five other *Entomoneis* species (*E. japonica*, *E. paludosa*, *E. punctulata*, *E. aequabilis* and *E. vertebralis*) are presented in Table 4. The new species, *Entomoneis tenera sp. nov.*, is morphologically similar to other species in the genus, having a panduriform frustule with a well-developed winged keel elevated from the valve face and the sigmoid raphe positioned on the keel apex in the raphe canal, numerous girdle bands and junction line. In addition, the following features are considered to be characteristic for the newly proposed species: (1) general appearance is very delicate with lightly silicified frustules, with no valve striation visible in LM, (2) the cells are much smaller than that of other species, (3) one multi-lobed plastid, (4) broad lanceolate valves with scalpeliform apices, (5) junction line straight to slightly arcuate, positioned at an angle of about 45° from the apical axis.

TABLE 4. Morphological features of *Entomoneis tenera sp. nov.* in comparison to similar species: *E. japonica*, *E. paludosa*, *E. punctulata*, *E. aequabilis* and *E. vertebralis*.

Feature	Similar species					New species
	E. japonica	***E. paludosa***	***E. punctulata***	***E. aequabilis***	***E. vertebralis***	***E. tenera sp. nov.***
LM & SEM						
General appearance	Cells solitary	Cells solitary	Cells solitary	Cells solitary	Cells solitary or united to form short chains	Cells solitary or form short chains in culture conditions
Plastid per cell	nd	Two axial plastids	nd	nd	Two plate-like plastids	One, multi-lobbed plate-like plastid
Frustule shape	Panduriform	Panduriform	Panduriform	Panduriform, longitudinally twisted	Panduriform	Panduriform, longitudinally twisted
Junction line	Bi-sinuous	Sinusoid	Arcuate, a short row of puncta, restricted to the end corner of the keel	np	np	Straight to slightly arcuate
Valve length	75–150 µm	40–130 µm	18–99 µm	47–57 µm	33–58 µm	11–22 µm

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TABLE 4. (Continued)

Feature	Similar species					New species
	E. japonica	***E. paludosa***	***E. punctulata***	***E. aequabilis***	***E. vertebralis***	***E. tenera sp. nov.***
Valve width	20–40 µm	20–50 µm	10–19 µm	7–9 µm	10–11 µm	3–7 µm
Valve striation	Parallel, biseriatae, 11–12 stria per 10 µm	Parallel, uniseriatae, 21–26 stria per 10 µm	Parallel, 34–36 stria per 10 µm	Oblique, 32–37 stria per 10 µm	np	Not visible in LM, in EM parallel, 30–50 stria per 10 µm on valve body, 18–42 per 10 µm on the keel
Valve apex	Acuminate	Acute	Acute	Broad scalpeliform	Acute	Scalpeliform
Valve shape	Linear-lanceolate	Broad linear	Broad linear	Linear, slightly sigmoid	Linear-lanceolate	Broad lanceolate
Keel shape	Strongly sigmoid	Sigmoid, slightly torsioned	Sigmoid	Strongly sigmoid	Sigmoid	Sigmoid, often strongly torsioned
TEM						
Raphe fibulae	+	+	+	+	+	+, 29–40 per 10 µm
Keel fibulae	At several levels	nd	np	np	At several levels, 19–22 per 10 µm	np
Basal fibulae	+	+	Several in apical corner	np	+	+, 5–6 per 1 µm
Striae perforation	Two rows of poroid areolae occluded by perforated hymen (hymenate pore occlusion)	One row of elliptical poroid areolae, closed externally with hymen, 18–25 per 10 µm within valve body stria, 22–40 areolae within keel stria per 10 µm	Hymen perforated with parallel marginal linear perforations	Hymen with perforations forming short lines, 40–45 per 1 µm near keel margin and 20–25 per 1 µm near the valve margin	np	Hymen with rectangular perforations arranged in two parallel lines along the stria edges; 26–37 per 1 µm near keel margin and 20–39 per 1 µm near the valve margin
No. of cingulum bands	5 open bands	5–6 open bands	5–6 open bands	5–6 open bands	4–6 unornamented bands	4–5 open bands

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TABLE 4. (Continued)

Feature	Similar species					New species
	E. japonica	***E. paludosa***	***E. punctulata***	***E. aequabilis***	***E. vertebralis***	***E. tenera sp. nov.***
Copula areolae	Two rows of areolae, abvalvar elongated, advalvar short	Two rows of poroids: abvalvar elongated advalvar circular	Two rows of areolae: Advalvar shorter than abvalvar in bands near the valve, almost equal in the abvalvar bands	Two rows of oblong areolae: advalvar elliptical/ round, abvalvar elongated, 46–57 per 10 µm	nd	Two rows of elongated areolae: 56–60 per 10 µm
Reference	Osada & Kobayasi 1985 (as *E. alata* var. *japonica*)	Osada & Kobayasi 1990c	Osada & Kobayasi 1990c	Osada & Kobayasi 1991	Clavero *et al* 1999	This study

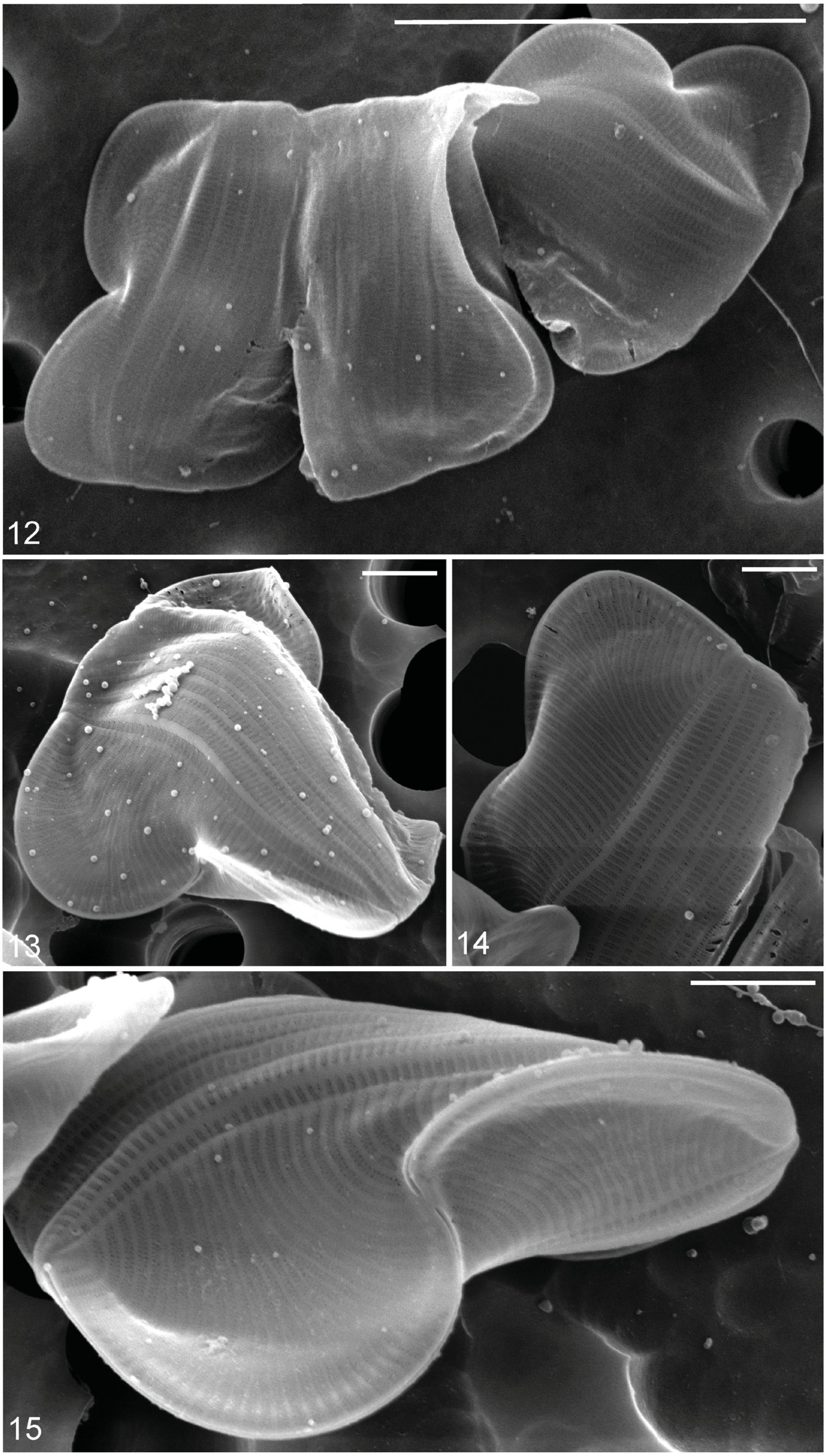
[*] nd: not defined; np: not present

Phylogenetic analyses

The ML trees generated from the combined nuclear SSU rDNA with chloroplast *rbc*L and *psb*C dataset and from the combined chloroplast genes alone are congruent with the respect to the phylogenetic positions of *E. tenera* strains PMFEN1, PMFEN2 and PMFEN3. In all phylogenetic analyses *Eunotia glacialis* (UTEX FD46) and *Eunotia pectinalis* (NIES461) were defined as an outgroup. SSU rDNA sequences of two strains (PMFEN1 and PMFEN2) were unfortunately not possible to obtain and thereby the first concatenated ML phylogenetic tree was generated using only the sequence obtained from strain PMFEN3 (Fig. 36). The second concatenated ML tree was based on all three *E. tenera* strains and both *rbc*L and *psb*C markers (Fig. 37). Both datasets resolved genus *Entomoneis* with *E. ornata* (14A), *Entomoneis* sp. (CS782) and *E. tenera* strains as monophyletic with strong support in SSU/CPL dataset (Bayesian posterior probability support/bootstrap support, BPP/BS=1.0/96) and weaker BS support in CPL dataset (BPP/BS=1.0/84) (Figs 36, 37). Both datasets also resolved Surirellales and Bacillariales as monophyletic with generally stronger support in the combined SSU/CPL dataset (BPP/BS=1.0/100and 1.0/79 respectively) than in CPL dataset alone (BPP/BS=1.0/100 and 1.0/61 respectively) (Figs 36, 37). In both datasets, Rhophalodiales are nested within Surirellales.

In both datasets the most closely related to our *E. tenera* strains appears to be *Entomoneis* sp. (CS782) (BPP/BS=1.0/100) while *E. ornata* branches off far from both CS782 and *E. tenera* strains (BPP/BS=1.0/96 in SSU/CPL and BPP/BS=1.0/84 in CPL dataset) (Figs 36, 37).

Phylogenetic analyses for individual datasets (SSU rDNA, *psb*C and *rbc*L) included more GenBank sequences for each gene to cover as much as possible cultivated and/or sequenced *Entomoneis* strains that exist in worldwide culture collections (see Electronic Supplement with Figs S1–S3). All three datasets revealed the same position of *E. tenera* regarding other *Entomoneis* species and clones with generally weaker BPP/BS support in SSU rDNA and *rbcL* datasets while in the *psb*C dataset BPP/BS support is quite similar to the concatenated CPL dataset (see Electronic Supplement with Figs S1, S2, S3). Finally, the last tested dataset (*rbc*L gene) showed the lowest tree backbone support with *E. tenera* clustering with CS782 (BPP/BS=0.59/-) and with *E. ornata* positioning far from all other *Entomoneis* species (see Electronic Supplement with Fig. S3).



FIGURES 12–15. *Entomoneis tenera* strain PMFEN2, SEM. Girdle view (Figs 12–14), valve view (Fig. 15). (12) Three cells attached with keels. (13) Cell twisted around the apical axis. (14) Girdle view of valve and cingulum with visible striation (costae bifurcation near the junction line indicated with an arrow). (15) Striation on the wing and valve body. Scale bars: Fig. 12=10 μm; Figs 13, 14, 15=2 μm.

DISCUSSION

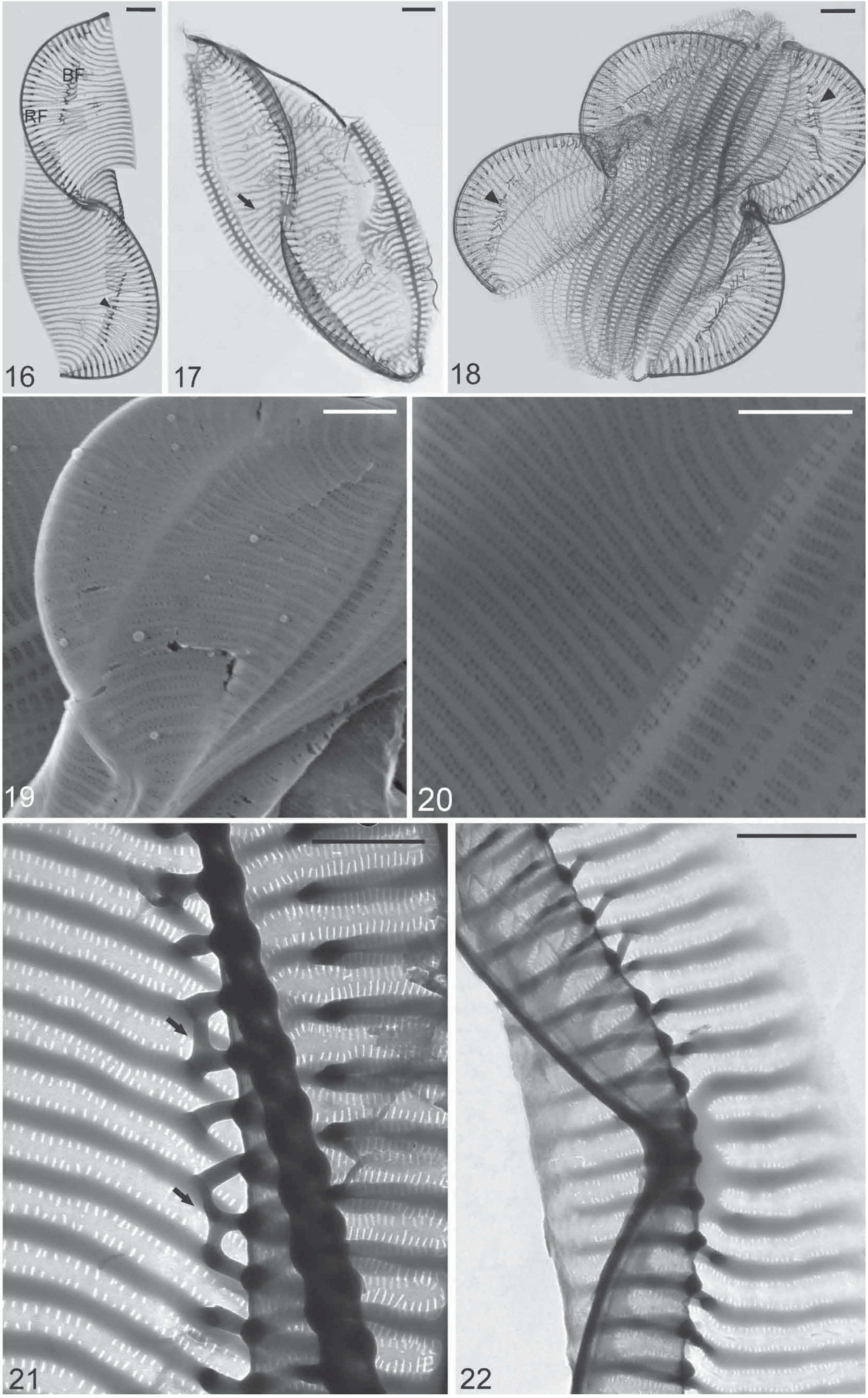
Based on its morphological characteristics such as panduriform shaped frustule, the raphe raised on a winged keel, junction line and numerous copulae, as well as the results of the molecular analyses from SSU, *rbc*L and *psb*C, *Entomoneis tenera sp. nov.* is unequivocally assigned to the genus *Entomoneis.* However, the new species exhibits several peculiar morphological features that will be discussed in comparison to other *Entomoneis* species.

Entomoneis tenera cells possess a single multi-lobed plastid unlike *E. paludosa* (Osada & Kobayasi 1990c), *E. vertebralis* (Clavero *et al.* 1999) or *E. reimerii* (Reinke & Wujek, 2013) that have two plastids per cell. Although Round *et al*. (1990) report that the species belonging to *Entomoneis* genus have one to two plastids, the authors are not aware of any particular record of a species with one plastid per cell. In most cases, species descriptions do not have a defined number of plastids at all, probably due to the fact that they were based solely on observation of cleaned material (Osada & Kobayasi 1985, 1990c, 1991). A particular feature of *Entomoneis tenera* is the minute size of its cells. The cell length of 16–21 µm is markedly shortest among described *Entomoneis* species when compared to 75–150 µm in *E. japonica,* 40–130 µm in *E. paludosa,* 18–99 µm *E. punctulata* or 47–57 µm in *E. aequabilis* (Osada & Kobayasi 1985, 1990c, 1991). The valve of *E. tenera* is also uniquely shaped, being broad lanceolate comparing to linear lanceolate shape reported for *E. japonica* and *E. vertebralis* (Osada & Kobayasi 1985, Clavero *et al*. 1999), broad linear for *E. paludosa* and *E. punctulata* (Osada & Kobayasi 1990c) and linear to slightly sigmoid in *E. aequabilis* (Osada & Kobayasi 1991).

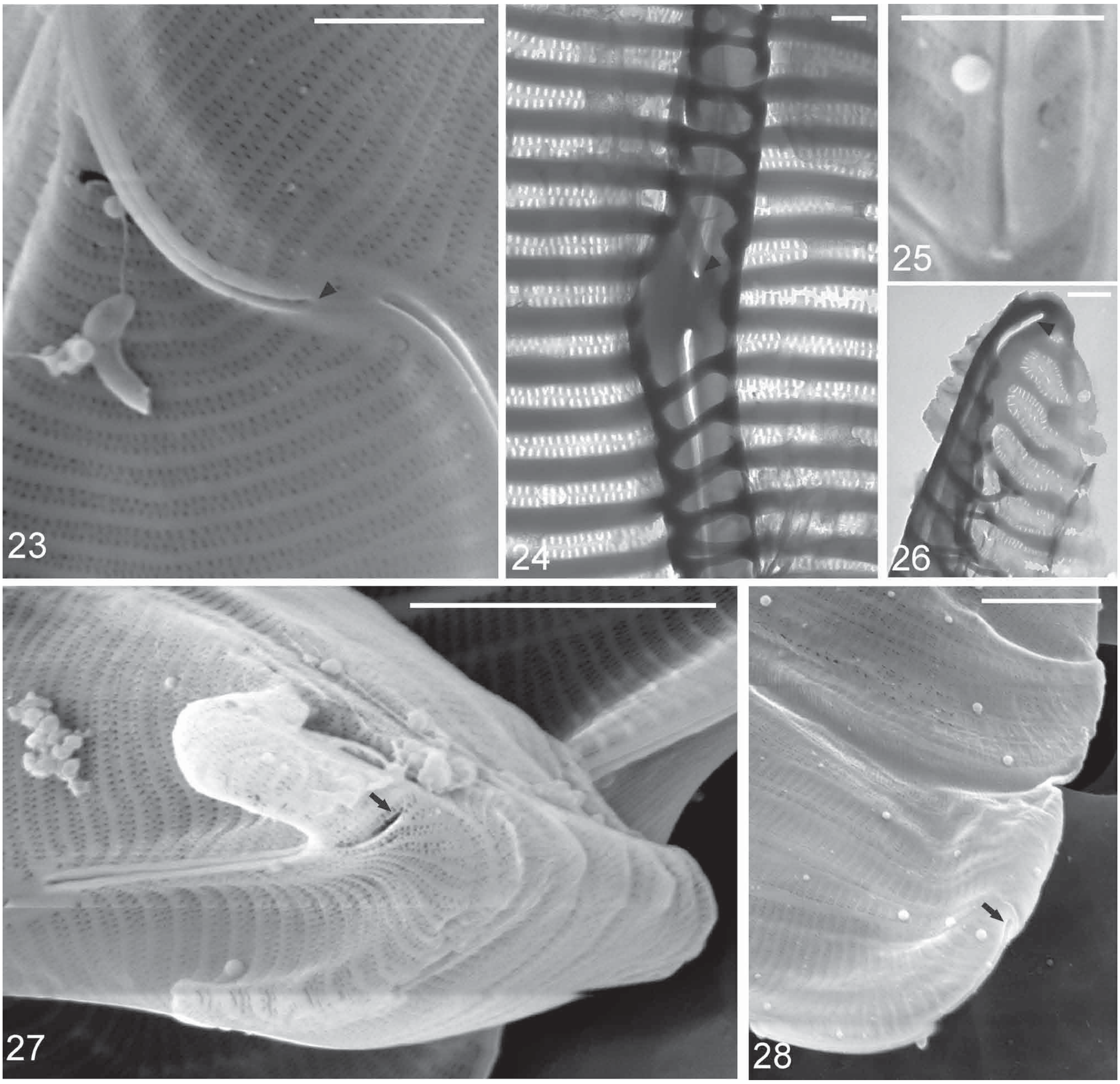
Another distinguishing feature of *E. tenera* is its delicate appearance due to the very weakly silicified cells, while other species generally appear more robust with more strongly silicified frustules. The only known exception is *Entomoneis vertebralis* that is described to have a "hyaline" frustule with raphe system as the only silicified part (Clavero *et al*. 1999). Although this species is the most similar to our *E. tenera,* with respect to the general appearance of the cells and the fact that there is no visible valve striation in LM, a careful EM examination of our taxon revealed the fine structure of the valve consisting of transapical costae and striae with rectangular perforations while in *E. vertebralis* there is no distinguishable ornamentation of the valves even with EM. Another resemblance of these two species is occasionally observed chain-like formation where the cells attach to each other with their keels (Clavero *et al*. 1999). The *Entomoneis* species are mostly solitary forms (Round *et al*. 1990) and together with the fact that the chain-like forms were only observed in laboratory cultures and never in field samples, we cannot consider these life-forms as a delineating feature of these species. It is highly possible that these "chains" were formed due to the lack of separation after cell division and not because these cells form true chain colonies in the natural habitat.

An important morphological feature of the *Entomoneis* genus, the junction of the keel with the valve body defined by a row of basal fibulae (Paddock & Sims 1981), is not always visible in our species due to weakly silicified cells, but it is possible to observe it in majority of specimens. The shape and position of junction line differ between *Entomoneis* species: bi-sinous in *E. japonica*, arcuate in *E. paludosa*, partially curved and restricted to the terminal corner of the wing in *E. punctulata*, inverted V-shape in *E. calixasini* to complete absence in *E. aequabilis* and *E. vertebralis* (Osada & Kobayasi 1985, 1990c, 1991, Clavero *et al*. 1999, Paillès *et al.* 2014). The junction line in *E. tenera* is straight to slightly arcuate and positioned at an angle of about 45° from the apical axis. Additionally, in *E. tenera,* adjacent basal fibulae that form a junction line are frequently connected with transverse connections forming a shape similar to letter H or W. The fused basal fibulae are also occasionally observed for *E. pseudoduplex* (Osada & Kobayasi 1990c), for *E. decussata* and *E. calixasini*, where they are H or Y shaped (Osada & Kobayasi 1990b, Paillès *et al.* 2014), and for *E. centrospinosa* where they are fishbone-like or H shaped (Osada & Kobayasi 1990a).

The raphe system in *E. tenera* is organized in a sigmoid raphe canal situated on keel apex which is separated with a row of raphe fibulae as in all other *Entomoneis* species. In our *E. tenera,* we cannot discriminate keel fibulae in contrast to *E. japonica* (Osada & Kobayasi 1985), *E. centrospinosa*, *E. decussata*, *E. pseudoduplex*, *E. vertebralis* (Clavero *et al*. 1999) and *E. calixasini* (Paillès *et al.* 2014). Also, the raphe slit opens in a single raphe canal whereas *E. paludosa* and *E. calixasini* have a double raphe canal (Paillès *et al.* 2014). Individual raphe fibulae in *E. tenera* are not discernible with LM as they are solely observable as a highly silicified continuous keel margin, whereas in *E. aequabilis*, *E. japonica* or *E. punctulata* the raphe fibulae are clearly defined as a small row of dash-like puncta along the keel margin (Osada & Kobayasi 1985, 1991, 1990c). *E. tenera* has a plicate raphe slit, similar to all described *Entomoneis* species (Osada Kobayasi 1985). Terminal and central raphe endings in *E. tenera* appear simple in their external part as for example in *E. aequabilis*, the opposite of *E. centrospinosa* and *E. calixasini*, where central endings are shaped as elongated droplets (Osada & Kobayasi 1991, 1985, 1990c, Paillès *et al.* 2014).

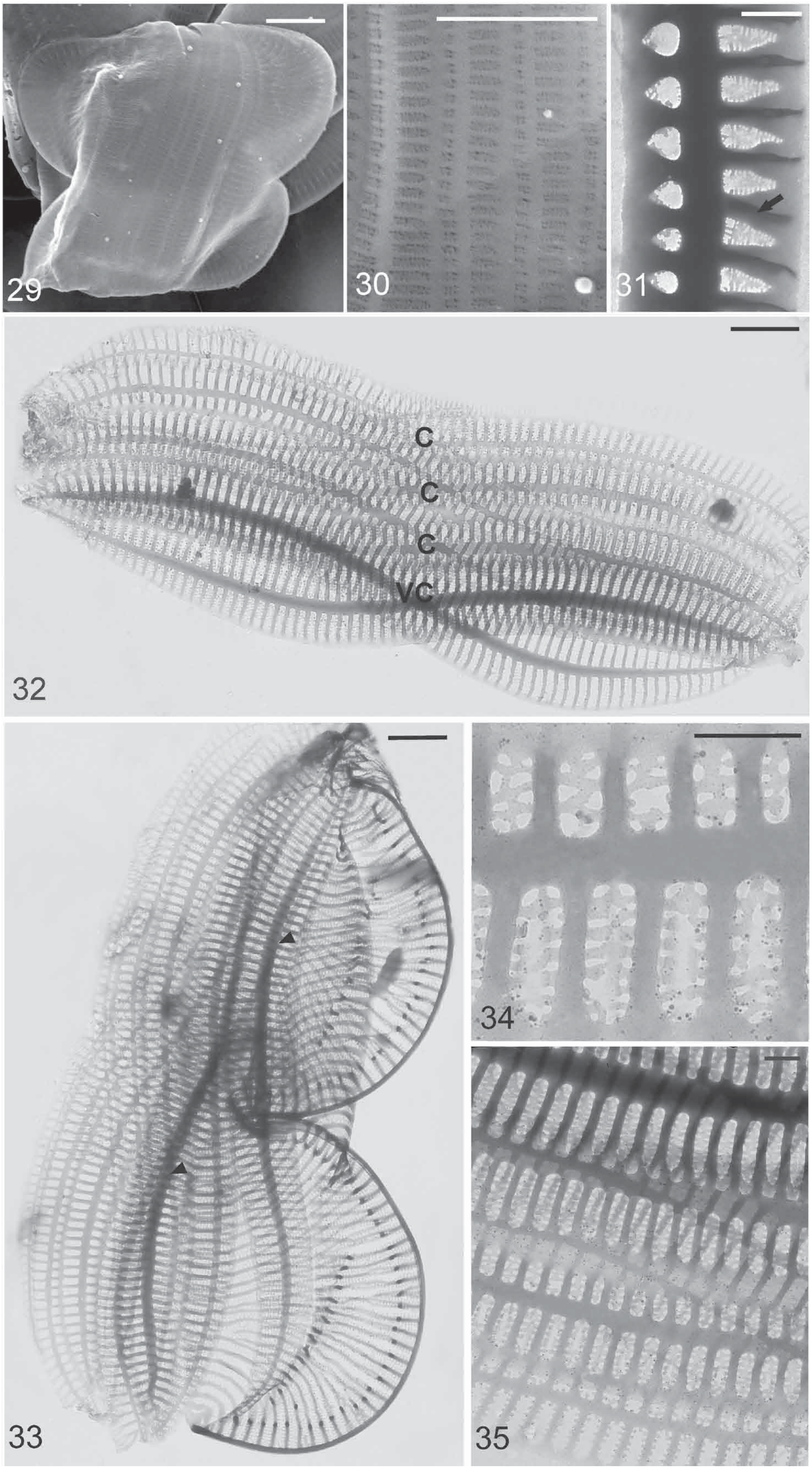


FIGURES 16–22. *Entomoneis tenera* strain PMFEN2, SEM and TEM. Girdle views (Figs 18–20), valve views (Figs 16, 17, 21, 22), RF-raphe fibulae, BF-basal fibulae (junction line). (16) Valve with scalpeliform apices and junction line (arrowhead). (17) Valve with valvocopulae and sigmoid raphe-bearing keel (costae bifurcations are indicated by arrow). (18) Cell with complete girdle and indicated junction lines (arrowheads). (19, 20) Fine structure of the wing and valve body. (21) Adjacent basal fibulae fused with transverse connections (arrows). (22) Basal fibulae separating wing from valve body. Scale bars=1 μm.



FIGURES 23–28 *Entomoneis tenera* strain PMFEN2, SEM and TEM. Girdle views (Figs 26, 28), valve views (Figs 23, 24. 25, 27). (23, 24) Central part of the valve with central node and simple central raphe endings (arrowhead). (25) Simple terminal raphe ending. (26) Valve apex with simple terminal raphe ending. (27) Partial view of the valve with simple central and apical raphe endings. (28) Girdle view of cell apex showing simple apical raphe ending (arrow) Scale bars: Fig. 27=2 µm; Figs 23, 25, 28=1 µm; Figs 24, 26=300 nm.

Fine ornamentation of *Entomoneis tenera* valves largely resembles the striation observed for *E. aequabilis* and *E. punctulata* (Osada & Kobayasi 1990c, 1991), where valve structure is ornamented by striae not composed of areolae *sensu stricto*, but rather a lightly silicified hymen is perforated by regular rectangular perforations. However, the density of the perforations differs among species with *E. tenera* having 26–37 per 1 µm near keel margin and 20–39 per 1 µm near the valve margin as opposed to *E. aequabilis* that has 40–45 perforations per 1 µm near keel margin and 20–25 per 1 µm near the valve margin (Osada & Kobayasi 1990c). Another peculiar ultrastructural feature is the occasionally observed bifurcations of transapical costae at the valve margin or near the junction between valve body and wing. These bifurcations are not unique for *E. tenera* but also present in *E. aequabilis*, *E. centrospinosa* and *E. japonica* (Osada & Kobayasi 1985, 1990a, 1991).



FIGURES 29–35. *Entomoneis tenera* strain PMFEN2, SEM and TEM. VC-valvocopula; C-copula. Girdle views (Figs 29–35). (29) Frustule with the girdle. (30) Fine structure of the copulae. (31) Fine structure of valvocopulae with teardrop shaped areolae and inter-areolae thickenings (arrow). (32) Cingulum. (33) Valve with cingulum and decussate appearance of the costae on the valve between valvocopulae (arrowhead) and junction line. (34, 35) Fine structure of copulae. Scale bars: Figs 29, 30=2 µm: Figs 32, 33=1 µm; Figs 31, 34, 35=300 nm.

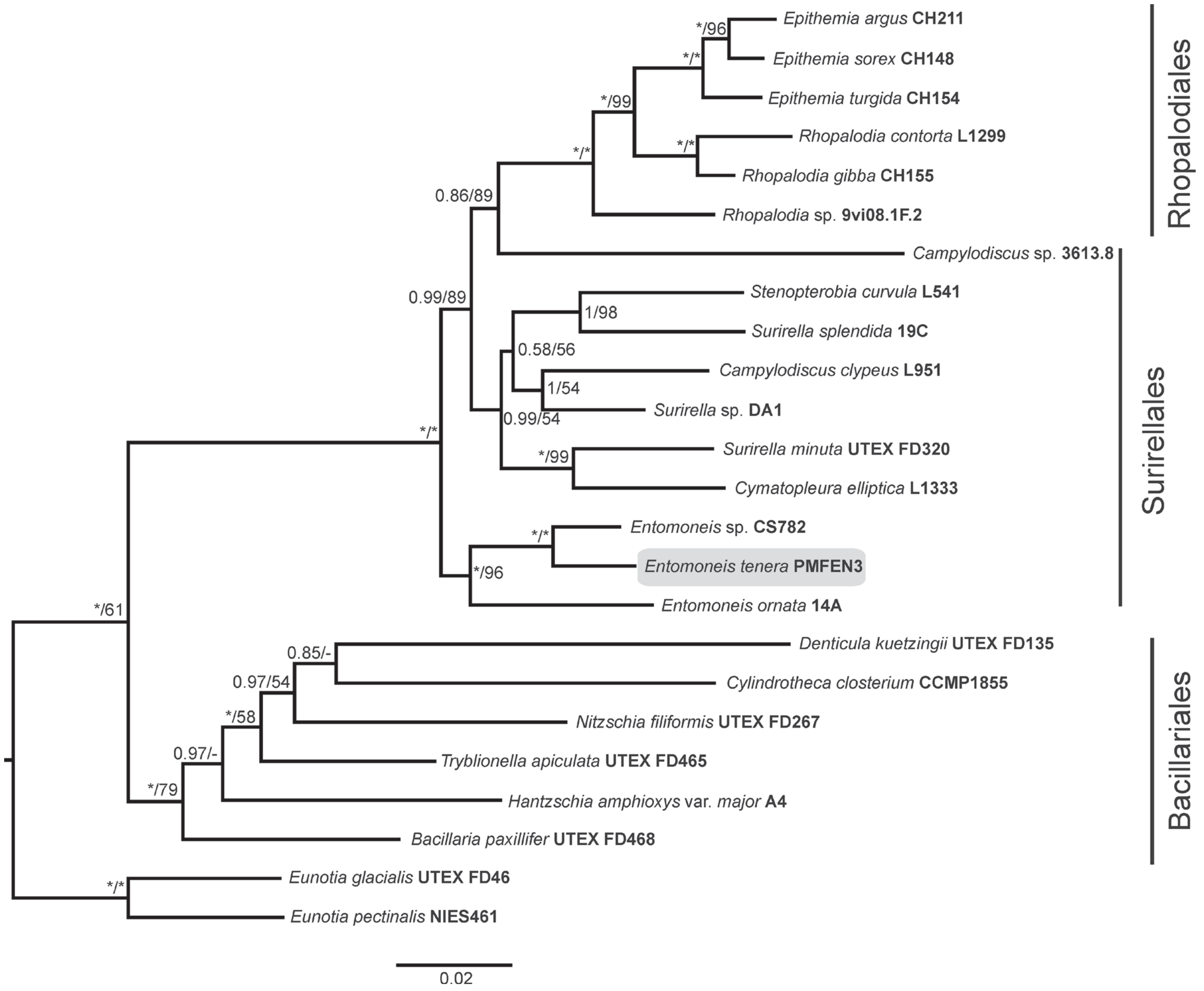


FIGURE 36. Maximum likelihood phylogram inferred from a concatenated dataset of three markers: SSU, *rbc*L and *psb*C. Branch support is summarized above branches as ML Bayesian posterior probabilities (BPP)/bootstrap values (BS). Taxon labels are indicated as name (in italic) strain (in bold)**.** Canal raphe bearing diatoms (Surirellales, Rhopalodiales and Bacilarialles) are indicated on the right side of phylogram. ML tree is based on GTR+G+I evolutionary model with 1,000 bootstrap replicates and 60M Bayesian generations. The tree is rooted with *Eunotia glacialis* (FD46) and *Eunotia pectinalis* (NIES461). BPP/BS values of 1.0/100 are indicated with asterisk (*) and values below 0.5/50 are indicated with dash (-).

Generally, the structure of the cingulum is similar among all *Entomoneis* species with numerous open porous bands (Round *et al*. 1990, Osada & Kobayasi 1985, 1990c, 1991). *E. tenera* is not an exception, with 4 to 5 open porous bands, perforated with two rows of areolae, opened and closed alternately at each frustule pole. Valvocopular areolae in *E. tenera* are elliptically shaped, abvalvar areolae with a smaller radius than advalvar ones. *E. japonica* and *E. aequabilis* have similar elongated abvalvar areolae but the advalvar ones are round to elliptical/round (Osada & Kobayasi 1985, 1991). The other important feature is the valvocopular areolae density which is higher in *E. tenera* (40–50 per 10 µm) than in other species, e. g. *E. pseudoduplex* 32–45 per 10 µm (Osada & Kobayasi 1990a); *E. decussata* 22–28 per 10 µm (Osada & Kobayasi 1990b) and *E. centrospina* 16–19 per 10 µm (Osada & Kobayasi 1990c). The only species which has more densely spaced areolae is *E. aequabilis* with 46–57 per 10 µm (Osada & Kobayasi 1991). In valvocopulae of several specimens, we also observed peculiar thickenings on the costae between unusual teardrop-shaped areolae. Similar structures can be compared to short I- or Y-shaped projections on the inter-areolae costae, described for *E. aequabilis* (Osada & Kobayasi 1991), but in these projections are positioned perpendicularly on the costae while in *E. tenera* thickenings position themselves parallel on the inter-areolar costae.

Based on multigene phylogeny, *E. tenera* branched with other *Entomoneis* species supporting the taxonomic affiliation of new species within this genus. In all analysed datasets, our strains grouped with *Entomoneis* sp. (CS782), an undescribed marine strain isolated from Dunalley, Tasmania, Australia (I. Jameson pers. comm.). In recent phylogenetic studies, the genus *Entomoneis* was represented with CS782 and *E. ornata* (14A), and both strains clustered together by strong bootstrap values (SSU, *rbc*L and *psb*C concatenated phylogeny/CPL phylogeny (BPP/BS=1.0/98; BPP/BS=1.0/91 respectively) in Ruck & Theriot (2011) and SSU, *rbc*L and *psb*C concatenated phylogeny (BPP/BS=1.0/89) in Witkowski *et al*. (2014). *Entomoneis* sp. strain CS782 is apparently morphologically very similar to *E. tenera* with respect to the general appearance and ultrastructure (E. Ruck and I. Jameson pers. comm.), but yet differs from our species as the frustule of CS782 is narrower and wings are more arcuate/straight than bilobate (Ruck *et al*. 2016). Results of the phylogenetic analyses (CPL dataset) show all three *E. tenera* strains forming a clade branching off CS782 (BPP/BS=1.0/100) confirming the morphological distinction between the species. This is corroborated by the results of the concatenated SSU/CPL phylogeny where CS782 and *E. tenera* are clearly separated with great BBP/BS value (1.0/100).

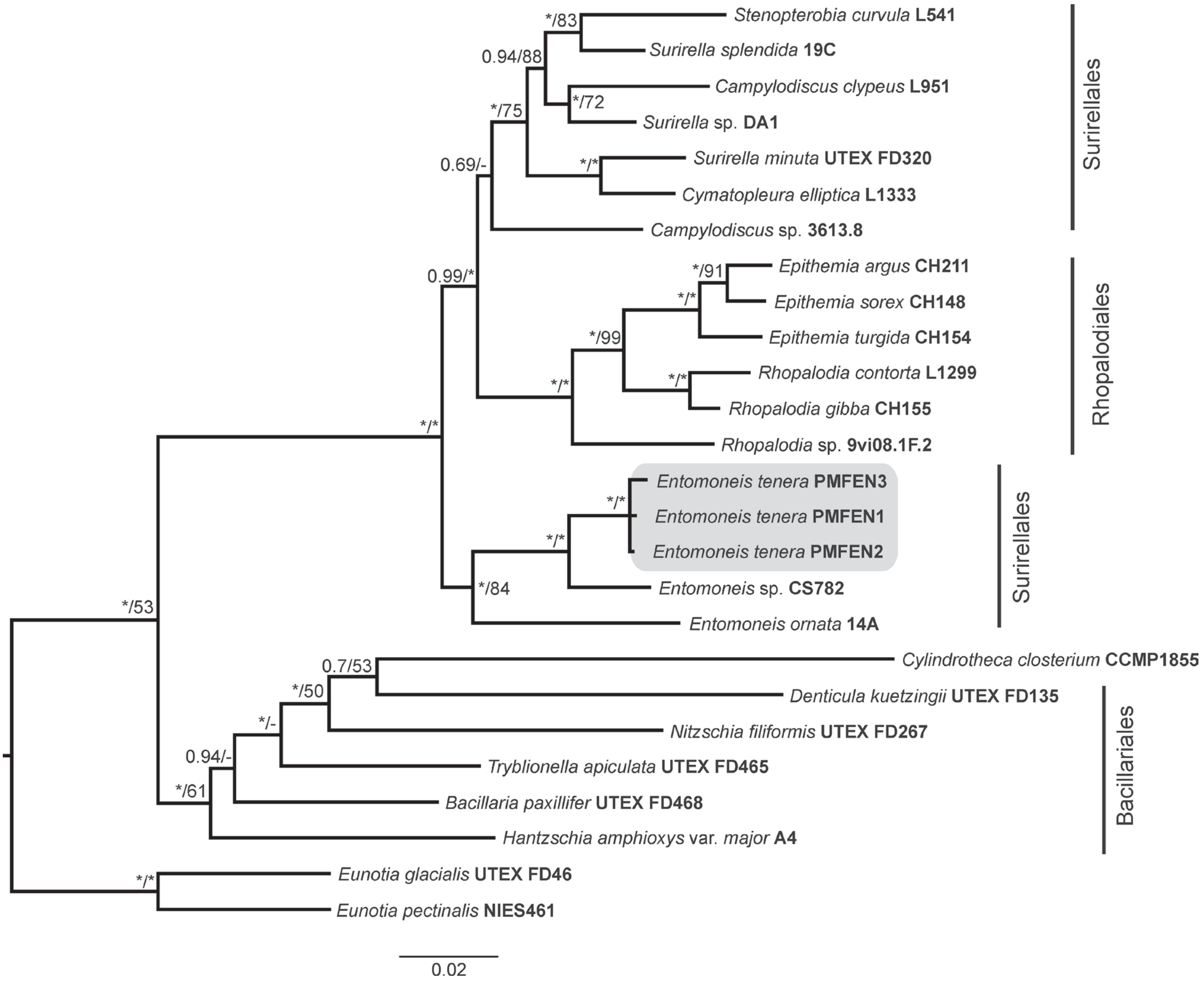


FIGURE 37. Maximum likelihood phylogram inferred from a concatenated dataset of two markers: *rbc*L and *psb*C. Branch support is summarized above branches as ML Bayesian posterior probabilities (BPP)/bootstrap values (BS). Taxon labels are indicated as name (in italic) strain (in bold). Canal raphe bearing diatoms (Surirellales, Rhopalodiales and Bacilariales) are indicated on the right side of phylogram. ML tree is based on GTR+G+I evolutionary model with 1,000 bootstrap replicates and 40M Bayesian generations. The tree is rooted with *Eunotia glacialis* (FD46) and *Eunotia pectinalis* (NIES461). BPP/BS values of 1.0/100 are indicated with asterisk (*) and values below 0.5/50 are indicated with dash (-).

A great deal of work remains to be done to explore the diversity within the genus *Entomoneis*, and this should be done taking into account both morphological and molecular information. The majority of currently described species are large, epipelic taxa, easy to study from the morphological perspective, however with scarce molecular data. Our study is the first study presenting a description of a novel species using combined morphological and molecular approach representing a starting point in exploration of the hidden diversity of the small planktonic species belonging to *Entomoneis* genus.

ACKNOWLEDGMENTS

This study was fully supported by Croatian Science Foundation under the project BIOTA UIP-11-2013-6433. Authors are grateful to J. Godrijan for isolation of PMFEN2 strain, E. Ruck, and I. Jameson (Australian National Algae Culture Collection) for providing information on the clone CS782, as well as to I. Stanković and "Hrvatske vode" for providing microscope facilities. The authors thank the editor and two anonymous reviewers for their helpful comments and suggestions as well as the participants at the 10th Central European Diatom Meeting in Budapest, Hungary, 20–23 April, 2016 on the fruitful discussions which greatly improved our manuscript.

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Electronic Supplement

Table S1. List of taxa included in this study with accession numbers for three different genes. Classification system follows Round *et al*. 1990.

Figure S1. Maximum likelihood phylogram inferred from SSU gene alignment. Branch support is summarized below branches as ML Bayesian posterior probabilities (BPP)/bootstrap values (BS). Taxon labels are indicated as name (in italic) strain (in bold). Canal raphe bearing diatoms (Surirellales, Rhopalodiales and Bacilarialles) are indicated on the right side of phylogram. ML tree is based on GTR+G+I evolutionary model with 1,000 bootstrap replicates and 15M Bayesian generations. The tree is rooted with *Eunotia glacialis* (FD46) and *Eunotia pectinalis* (NIES461). BPP/BS values of 1.0/100 are indicated with asterisk (*) and values below 0.5/50 are indicated with dash (-).

Figure S2. Maximum likelihood phylogram inferred from psbC gene alignment. Branch support is summarized below branches as ML Bayesian posterior probabilities (BPP)/bootstrap values (BS). Taxon labels are indicated as name (in italic) strain (in bold). Canal raphe bearing diatoms (Surirellales, Rhopalodiales and Bacilarialles) are indicated on the right side of phylogram. ML tree is based on GTR+G+I evolutionary model with 1,000 bootstrap replicates and 15M Bayesian generations. The tree is rooted with *Eunotia glacialis* (FD46) and *Eunotia pectinalis* (NIES461). BPP/BS values of 1.0/100 are indicated with asterisk (*) and values below 0.5/50 are indicated with dash (-).

Figure S3. Maximum likelihood phylogram inferred from rbcL gene alignment. Branch support is summarized below branches as ML Bayesian posterior probabilities (BPP)/bootstrap values (BS). Taxon labels are indicated as name (in italic) strain (in bold). Canal raphe bearing diatoms (Surirellales, Rhopalodiales and Bacilarialles) are indicated on the right side of phylogram. ML tree is based on GTR+G+I evolutionary model with 1,000 bootstrap replicates and 15M Bayesian generations. The tree is rooted with *Eunotia glacialis* (FD46) and *Eunotia pectinalis* (NIES461). BPP/BS values of 1.0/100 are indicated with asterisk (*) and values below 0.5/50 are indicated with dash (-).

Table S1. List of taxa included in this study with accession numbers for three different genes. Classification system follows Round *et al*. 1990.

Taxon	**Culture ID**	**SSU**	**rbcL**	**psbC**	**Reference**
Amphiprora alata (Ehr.) Kützing = *Entomoneis alata* (Ehr.) Ehrenberg	C108	FJ002099.1			Rampen *et al*., direct submission
Amphiprora paludosa var. *hyalina* (Eulenstein ex Van Heurck) Cleve = *Amphiprora hyalina* Eulenstein ex Van Heurck	CCAP1003/4	FR865482.1			Heesch, S., direct submission
Amphiprora paludosa W. Smith = *Entomoneis paludosa* (W.Smith) Reimer	CCMP125	AY485468.1			Damsté *et al*. 2004
Amphiprora paludosa W. Smith = *Entomoneis paludosa* (W.Smith) Reimer	C52	FJ002140.1			Rampen *et al*., direct submission
Bacillaria paxillifer (O. Müll.) Hendey	FD468	HQ912627.1	HQ912491.1	HQ912320.1	Theriot *et al*. 2010
Campylodiscus clypeus Ehrenberg	L951	HQ912412.1	HQ912398.1	HQ912384.1	Ruck & Theriot 2011
Campylodiscus sp.	3613.8	HQ912413.1	HQ912399.1	HQ912385.1	Ruck & Theriot 2011
Cylindrotheca closterium (Ehr.) Reimann et Lewin	CCMP1855	HQ912645.1	HQ912509.1	HQ912338.1	Theriot *et al*. 2010
Cymatopleura elliptica (Bréb.) W. Smith	L1333	HQ912659.1	HQ912523.1	HQ912352.1	Theriot *et al*. 2010
Denticula kuetzingii Grunow	FD135	HQ912610.1	HQ912474.1	HQ912303.1	Theriot *et al*. 2010
Entomoneis cf. *alata*	p540	AJ535160.1			Medlin & Kaczmarska 2004
Entomoneis cf. *alata*	-	AY534908.1			Damsté *et al*. 2004

Entomoneis cf. *alata*	C99	FJ002100.1			Rampen *et al*., direct submission
Entomoneis ornata (Bailey) Reimer in Patrick & Reimer	14A	HQ912411.1	HQ912397.1	HQ912383.1	Ruck & Theriot 2011
Entomoneis pseudoduplex Osada & Kobayasi = *Amphiprora pseudoduplex* (Osada & Kobayasi) Hällfors	ROS_AF18	KP400266.1	KP400299.1		Stachura-Suchoples *et al*. 2015
Entomoneis pseudoduplex Osada & Kobayasi = *Amphiprora pseudoduplex* (Osada & Kobayasi) Hällfors	ROS_AF18*	KP400267.1	KP400300.1		Stachura-Suchoples *et al*. 2015
Entomoneis pseudoduplex Osada & Kobayasi = *Amphiprora pseudoduplex* (Osada & Kobayasi) Hällfors	ROS_KD16	KP400277.1	KP400303.1		Stachura-Suchoples *et al*. 2015
Entomoneis pseudoduplex Osada & Kobayasi = *Amphiprora pseudoduplex* (Osada & Kobayasi) Hällfors	ROS_KD19	KP400280.1	KP400304.1		Stachura-Suchoples *et al*. 2015
Entomoneis punctulata (Grun.) K.Osada & H. Kobayasi	BA83	HM805031.1			Pniewski *et al*. 2011
Entomoneis sp.	CCMP2396	KF899836.1			Gilg, I. & Preston, M.J., direct submission
Entomoneis sp.	CS782	HQ912631.1	HQ912495.1	HQ912324.1	Theriot *et al*. 2010
Entomoneis sp.	CCMP1693	EF585586.1			Sorhannus 2007
Entomoneis sp.	RCC2678	KT861118.1			LeGall, F. *et al*., direct submission
Entomoneis sp.	RCC4487	KT878709.1			Gourvil, P. & Vaulot, D., direct submission
Epithemia argus (Ehr.) Kützing	CH211	HQ912408.1	HQ912394.1	HQ912380.1	Ruck & Theriot 2011

Epithemia sorex Kützing	CH148	HQ912409.1	HQ912395.1	HQ912381.1	Ruck & Theriot 2011
Epithemia turgida (Ehr.) Kützing	CH154	HQ912410.1	HQ912396.1	HQ912382.1	Ruck & Theriot 2011
*Eunotia glacialis*Meister	FD46	HQ912586.1	HQ912450.1	HQ912279.1	Theriot *et al*. 2010
Eunotia pectinalis (Kütz.) Rabenhorst	NIES461	HQ912636.1	HQ912500.1	HQ912329.1	Theriot *et al*. 2010
Hantzschia amphioxys var. *major* Grun. in Van Heurck	A4	HQ912404.1	HQ912390.1	HQ912376.1	Ruck & Theriot 2011
Nitzschia filiformis (W. Sm.) Van Heurck	FD267	HQ912589.1	HQ912453.1	HQ912282.1	Theriot *et al*. 2010
Rhopalodia contorta Hustedt	L1299	HQ912406.1	HQ912392.1	HQ912378.1	Ruck & Theriot 2011
Rhopalodia gibba (Ehr.) O. Müller	CH155	HQ912407.1	HQ912393.1	HQ912379.1	Ruck & Theriot 2011
Rhopalodia sp.	9vi08.1F.2	HQ912405.1	HQ912391.1	HQ912377.1	Ruck & Theriot 2011
Stenopterobia curvula (W. Sm.) Krammer	L541	HQ912416.1	HQ912402.1	HQ912388.1	Ruck & Theriot 2011
Surirella minuta	FD320	HQ912658.1	HQ912522.1	HQ912351.1	Theriot *et al*. 2010
Surirella sp. (Fastuosaegroup)	DA1	HQ912414.1	HQ912400.1	HQ912386.1	Ruck & Theriot 2011
Surirella splendida Ehrenberg	19C	HQ912415.1	HQ912401.1	HQ912387.1	Ruck & Theriot 2011
Tryblionella apiculata Greg.	FD465	HQ912600.1	HQ912464.1	HQ912293.1	Theriot *et al*. 2010

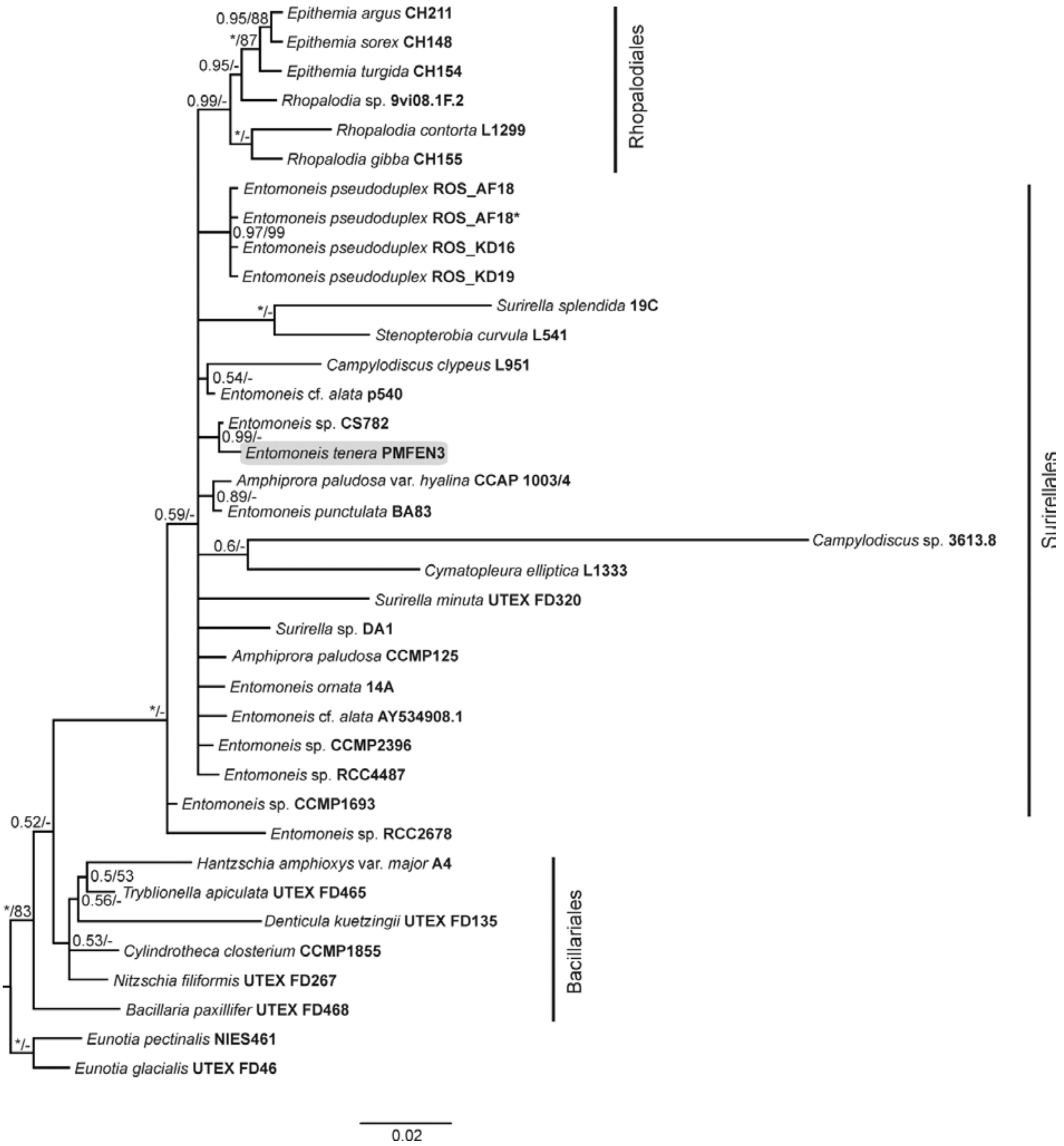
0.95/88
Epithemia argus CH211
*/87
Epithemia sorex CH148
0.95/-
Epithemia turgida CH154
0.99/-
Rhopalodia sp. 9vi08.1F.2
*/-
Rhopalodia contorta L1299
Rhopalodia gibba CH155
Rhopalodiales
Entomoneis pseudoduplex ROS_AF18
Entomoneis pseudoduplex ROS_AF18*
0.97/99
Entomoneis pseudoduplex ROS_KD16
Entomoneis pseudoduplex ROS_KD19
*/-
Surirella splendida 19C
Stenopterobia curvula L541
Campylodiscus clypeus L951
0.54/-
Entomoneis cf. alata p540
Entomoneis sp. CS782
0.99/-
Entomoneis tenera PMFEN3
Amphiprora paludosa var. hyalina CCAP 1003/4
0.89/-
Entomoneis punctulata BA83
0.59/-
0.6/-
Campylodiscus sp. 3613.8
Cymatopleura elliptica L1333
Surirella minuta UTEX FD320
Surirella sp. DA1
Amphiprora paludosa CCMP125
Entomoneis ornata 14A
*/-
Entomoneis cf. alata AY534908.1
Entomoneis sp. CCMP2396
Entomoneis sp. RCC4487
Entomoneis sp. CCMP1693
0.52/-
Entomoneis sp. RCC2678
Surirellales
Hantzschia amphioxys var. major A4
0.5/53
Tryblionella apiculata UTEX FD465
0.56/-
*/83
Denticula kuetzingii UTEX FD135
0.53/-
Cylindrotheca closterium CCMP1855
Nitzschia filiformis UTEX FD267
Bacillaria paxillifer UTEX FD468
Bacillariales
*/-
Eunotia pectinalis NIES461
Eunotia glacialis UTEX FD46
0.02

FIGURE S1.

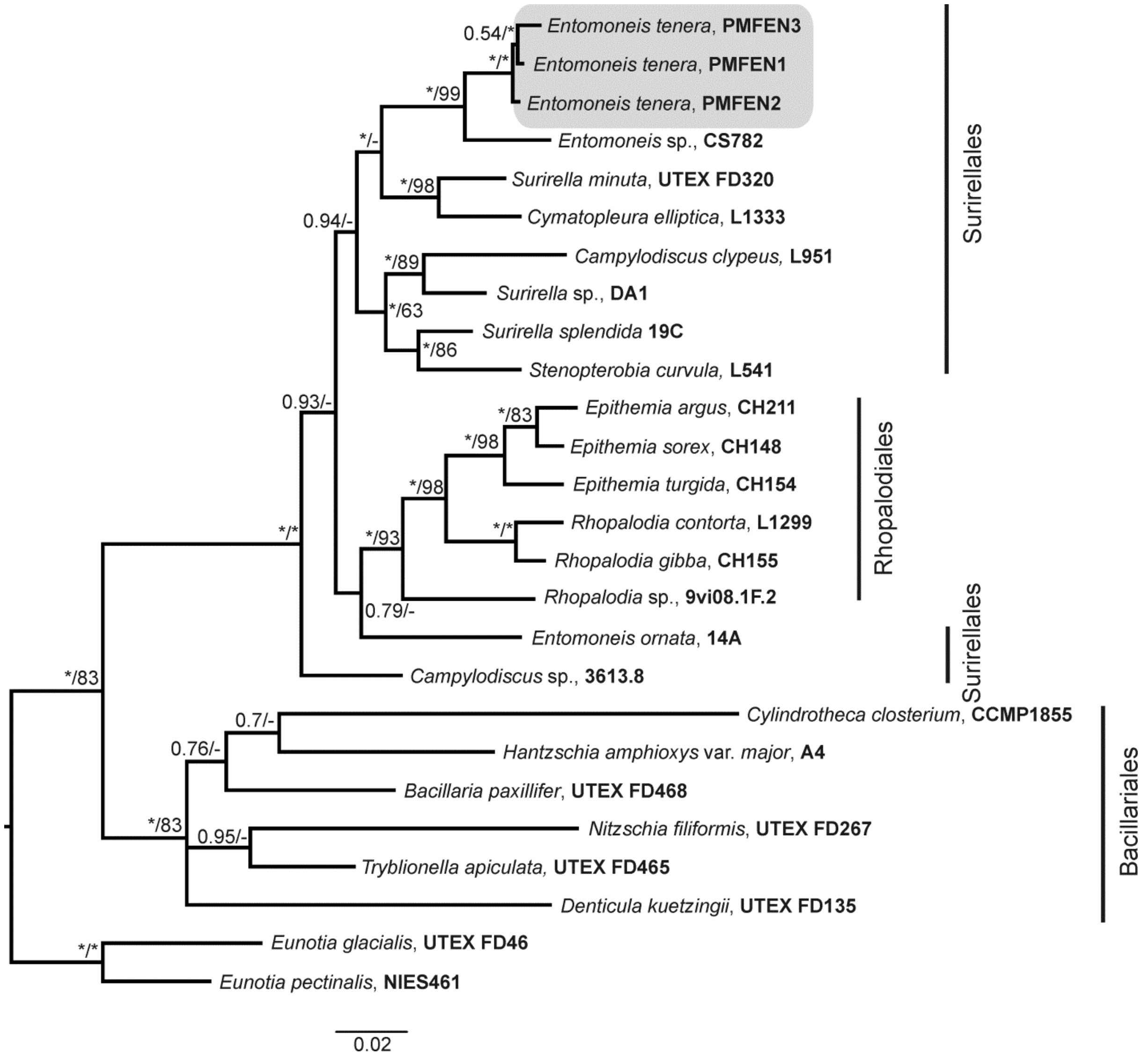
0.54/*
Entomoneis tenera, PMFEN3
/
Entomoneis tenera, PMFEN1
*/99
Entomoneis tenera, PMFEN2
*/-
Entomoneis sp., CS782
*/98
Surirella minuta, UTEX FD320
0.94/-
Cymatopleura elliptica, L1333
*/89
Campylodiscus clypeus, L951
Surirella sp., DA1
*/63
Surirella splendida 19C
*/86
Stenopterobia curvula, L541
Surirellales
0.93/-
*/83
Epithemia argus, CH211
*/98
Epithemia sorex, CH148
*/98
Epithemia turgida, CH154
/
Rhopalodia contorta, L1299
*/93
Rhopalodia gibba, CH155
Rhopalodia sp., 9vi08.1F.2
0.79/-
Entomoneis ornata, 14A
Rhopalodiales
Campylodiscus sp., 3613.8
Surirellales
*/83
0.7/-
Cylindrotheca closterium, CCMP1855
0.76/-
Hantzschia amphioxys var. major, A4
Bacillaria paxillifer, UTEX FD468
*/83
0.95/-
Nitzschia filiformis, UTEX FD267
Tryblionella apiculata, UTEX FD465
Denticula kuetzingii, UTEX FD135
Bacillariales
/
Eunotia glacialis, UTEX FD46
Eunotia pectinalis, NIES461
0.02

FIGURE S2.

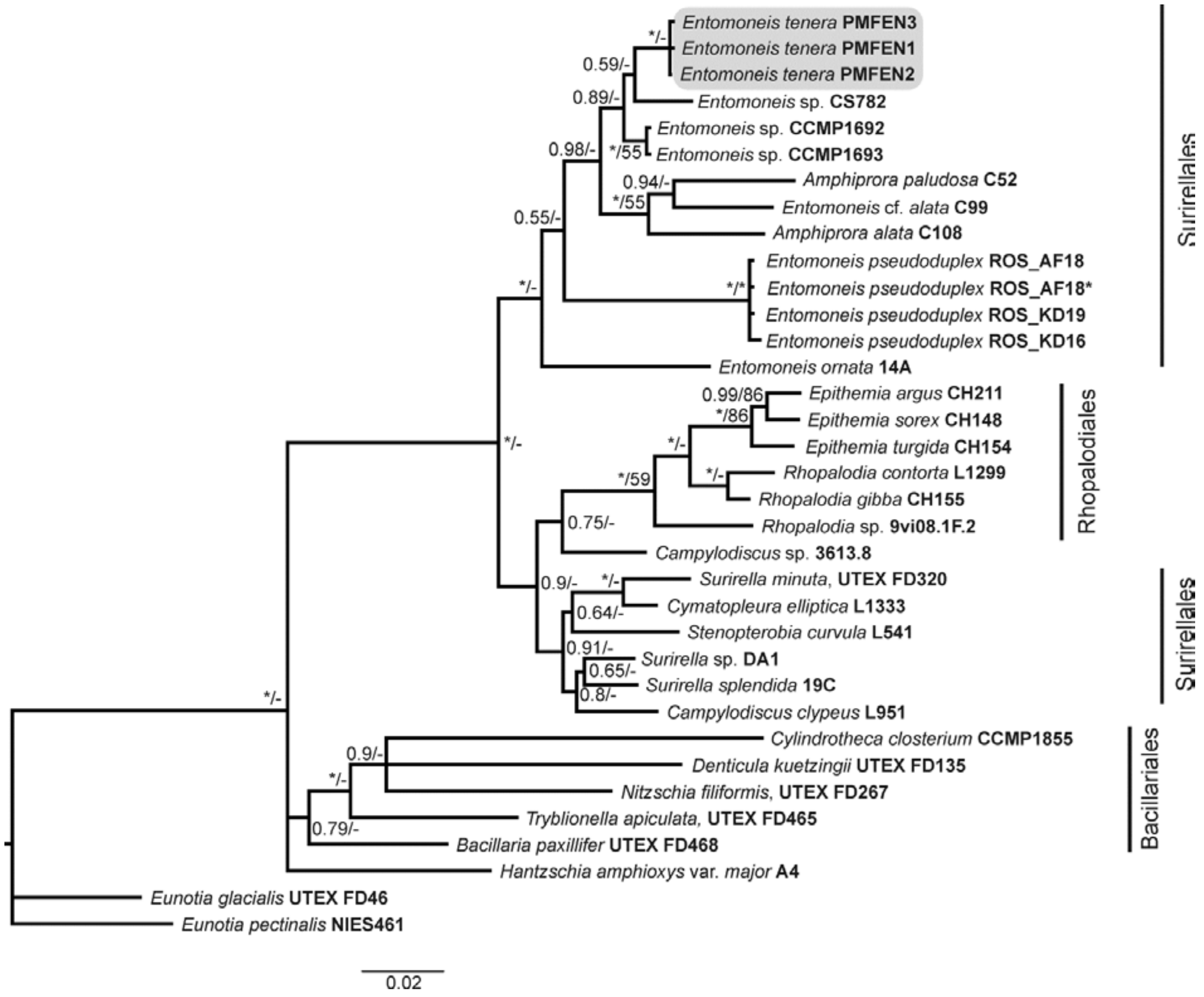
Entomoneis tenera PMFEN3
Entomoneis tenera PMFEN1
Entomoneis tenera PMFEN2
Entomoneis sp. CS782
Entomoneis sp. CCMP1692
Entomoneis sp. CCMP1693
Amphiprora paludosa C52
Entomoneis cf. alata C99
Amphiprora alata C108
Entomoneis pseudoduplex ROS_AF18
Entomoneis pseudoduplex ROS_AF18*
Entomoneis pseudoduplex ROS_KD19
Entomoneis pseudoduplex ROS_KD16
Entomoneis ornata 14A
Epithemia argus CH211
Epithemia sorex CH148
Epithemia turgida CH154
Rhopalodia contorta L1299
Rhopalodia gibba CH155
Rhopalodia sp. 9vi08.1F.2
Campylodiscus sp. 3613.8
Surirella minuta, UTEX FD320
Cymatopleura elliptica L1333
Stenopterobia curvula L541
Surirella sp. DA1
Surirella splendida 19C
Campylodiscus clypeus L951
Cylindrotheca closterium CCMP1855
Denticula kuetzingii UTEX FD135
Nitzschia filiformis, UTEX FD267
Tryblionella apiculata, UTEX FD465
Bacillaria paxillifer UTEX FD468
Hantzschia amphioxys var. major A4
Eunotia glacialis UTEX FD46
Eunotia pectinalis NIES461
*/-
0.59/-
0.89/-
*/55
0.98/-
0.94/-
*/55
0.55/-
/
*/-
*/-
0.99/86
*/86
*/-
*/59
*/-
0.75/-
0.9/-
*/-
0.64/-
0.91/-
0.65/-
0.8/-
*/-
0.9/-
*/-
0.79/-
0.02
Surirellales
Rhopalodiales
Surirellales
Bacillariales

FIGURE S3.

Publication II

J. Phycol. **54,** 275–298 (2018)

DOI: 10.1111/jpy.12622

MORPHOLOGICAL DIVERSITY AND PHYLOGENY OF THE DIATOM GENUS *ENTOMONEIS* (BACILLARIOPHYTA) IN MARINE PLANKTON: SIX NEW SPECIES FROM THE ADRIATIC SEA[1]

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The diatom genus *Entomoneis* is known from the benthos and plankton of marine, brackish, and freshwaters. *Entomoneis* includes diatoms with a bilobate keel elevated above the valve surface, a sigmoid canal raphe, and numerous girdle bands. Owing mostly to the scarcity of molecular data for a diverse set of species, the phylogeny of *Entomoneis* has not been investigated in depth. The few previous studies that included *Entomoneis* were focused on broader questions and the available data were from a small number of either unidentified *Entomoneis* or well-known species (e.g., *E. paludosa*). Since the first description of new species combining both molecular and morphological characters (*E. tenera*), we have continued to cultivate and investigate *Entomoneis* in the plankton of the Adriatic Sea. Combined multigene phylogeny (SSU rDNA sequences, *rbc*L, and *psb*C genes) and morphological observations (LM, SEM and TEM) revealed six new *Entomoneis* species supported by phylogenetic and morphological data: *E. pusilla*, *E. gracilis*, *E. vilicicii*, *E. infula*, *E. adriatica*, and *E. umbratica*. The most important morphological features for species delineation were cell shape, the degree and mode of torsion, valve apices, the appearance and structure of the transition between keel and valve body, the ultrastructure and the shape of the girdle bands, and the arrangement and density of perforations along the valve and valvocopulae. Our results highlight the underappreciated diversity of *Entomoneis* and call for a more in-depth morphological and molecular investigation of this genus especially in planktonic habitats.

Key index words: ***Entomoneis*****; diatoms; Adriatic Sea; phytoplankton; morphology; phylogeny**

Abbreviations**: BI, Bayesian inference; ML, maximum likelihood; SSU, small ribosomal subunit; *rbc*L, ribulose–1,5–bisphosphate carboxylase/oxygenase large subunit; *psb*C, photosystem II CP43 protein**

Diatoms (Bacillariophyta) are photoautotrophic heterokonts with intricately ornamented siliceous

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[1] Received 9 June 2017. Accepted 17 January 2018. First Published Online 8 February 2018. Published Online 7 March 2018, Wiley Online Library (wileyonlinelibrary.com).

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Editorial Responsibility: P. Kroth (Associate Editor)

cell walls. They constitute one of the most abundant and diverse lineages in planktonic, benthic, and terrestrial habitats worldwide, with estimates of up to 200,000 species (Mann and Droop 1996). The "200,000 hypothesis", although widely cited (e.g., Alverson 2008, Vanormelingen et al. 2008, Armbrust 2009, Rynearson et al. 2009) is still debated among phycologists. Guiry (2012) gave a conservative figure of 12,000 described species of diatoms and 8,000 yet to be discovered, whereas Mann and Vanormelingen (2013) estimated at least 30,000 but possibly up to 100,000 species. Regardless of which of these estimates are more accurate, there is no doubt that a large fraction of diatom diversity is yet to be described, or even collected. Bridging this gap would require biodiversity studies that take advantage of all relevant information, including morphological, molecular, physiological, ecological, and biogeographic data. Global ocean sampling expeditions, like the *Tara* Oceans, Malaspina and others, are beginning to bridge this gap by generating large amounts of genetic and phenotypic data and uncovering biodiversity patterns at global scales (Nealson and Venter 2007, Agusti et al. 2015, De Vargas et al. 2015). These studies have discovered novel diversity around species and genera which historically have been considered relatively species poor (e.g., *Planktoniella*; Malviya et al. 2016) and suggest that there are likely many other groups of planktonic diatoms that are similarly understudied.

Species belonging to the genus *Entomoneis* share a sigmoid raphe positioned on a bilobate keel, a wing-like elevation that gives frustules panduriform shape in the girdle view, and numerous girdle bands. Means for discerning species are provided by cell size, the number and shape of chloroplasts, differences in valve shape and the transition between the keel and valve body. In many cases, however, reliable identification requires scanning (SEM) and or transmission (TEM) electron microscopy of the ultrastructural features such as variations in valve and girdle band perforation. Of the 46 *Entomoneis* species and intraspecific taxa currently included in Algaebase, only 19 species are considered taxonomically valid (Guiry and Guiry 2017). Within the last two decades, one fossil and four extant *Entomoneis* species have been described (Clavero et al. 1999, Cremer et al. 2003, Reinke and Wujek 2013, Paillès et al. 2014), and of these, the description of *Entomoneis tenera* pioneered the use of combined morphological and molecular evidence for species delimitation within the *Entomoneis* genus (Mejdandžić et al. 2017).

Round et al. (1990) and Patrick and Reimer (1975) defined *Entomoneis* as an epipelic genus inhabiting mainly marine and brackish sediments with rare occurrences in freshwaters. From today's perspective, the range of habitats where *Entomoneis* can be found is broader and includes both benthic and planktonic habitats from cold polar regions to warm tropical seas and inland waters of various salinities (Sutherland 2008, Dalu et al. 2015). In some cases, ecological preferences are well understood. For example, *Entomoneis paludosa* (prefers habitats with conductivity and pH of 3.28 and 8.6 $\mu S \cdot cm^{-1}$, respectively; Dalu et al. 2015). *Entomoneis vertebralis* and *E. reimeri* are typically found in natural salt marshes (Clavero et al. 1999, Reinke and Wujek 2013). *Entomoneis gigantea* and *E. kjellmanii* are sea ice species; the latter can be dominant in polar regions and especially abundant in association with platelet ice (McMinn and Hodgson 1993, Poulin et al. 2006). *Entomoneis ornata* tolerates eutrophic conditions and slightly brackish waters, but has also been recorded from freshwater lakes (Poulin and Cardinal 1982, Kociolek 2005, Carter and Belcher 2010). Overall, *Entomoneis* diversity in nonmarine environments is relatively poor, with marine and brackish taxa accounting for the majority of records from low salinities (e.g., *E. paludosa*, *E. paludosa* var. *duplex*, *E. ornata*; Osada and Kobayasi 1990c, Dalu et al. 2015). The marine plankton appears to harbour an underappreciated *Entomoneis* diversity (Paillès et al. 2014, Błachowiak-Samołyk et al. 2015, Mejdandžić et al. 2017) and majority of species are understudied from a taxonomic and phylogenetic perspective. Understanding the phylogeny of *Entomoneis* has been hindered by the fact that the majority of available molecular sequences originate from strains that have not been identified to the species level (e.g., Damsté et al. 2004, Medlin and Kaczmarska 2004, Moniz and Kaczmarska 2009, Ruck et al. 2016).

In this study, we contribute toward one of these knowledge gaps – the diversity of *Entomoneis* in temperate planktonic habitats – by studying the morphology and reconstructing the phylogeny for 12 strains isolated from the Adriatic Sea. We used light and electron microscopy to describe frustule morphology, and we sequenced three genes to infer phylogenetic relationships between newly isolated Adriatic strains and previously sequenced species. Although working in a geographically small and relatively confined area, we found considerable morphological variability among *Entomoneis* isolates, which led to the description of six new species supported by morphological and molecular data.

MATERIALS AND METHODS

Cultures. Samples containing *Entomoneis* cells were collected from Adriatic Sea with phytoplankton net (20 μm pore-size mesh) and 5 L Niskin bottles in June and October 2015 at station T2 (43°52′ N, 15°10′ E) and in March 2016 at stations P150 (42°32′ N, 17°59′ E); P600 (42°24′ N, 17°55′ E) and P1000 (42°20′ N, 17°49′ E). Samples collected with Niskin bottles were taken at various depths (30, 100, 150, 250 m). Both net and bottle samples were immediately inoculated in 0.22 μm filtered seawater taken from the collection site and enriched with f/2 nutrients (Guillard's f/2 Marine Water Enrichment Solution; Sigma-Aldrich, Gillingham, UK). Monoclonal cultures of 12 different strains: PMFT2EN2, PMFBIOP1,

PMFBION4A, PMFBION4B, PMFBION4C, BIOTAII–21, BIOTAII–41, BIOTAII–49, BIOTAII–60a, BIOTAII–68, BIOTAII–96, and BIOTAII–113 were obtained by micropipette isolation from enrichment samples under light microscope (Olympus CKX41; Olympus, Tokyo, Japan). Strains were maintained in plastic culture flasks (Jet Biofil®, Guangzhou, China) in 30 mL of f/2 liquid medium and transferred approximately weekly over the period of 8 months. Culture conditions were: temperature 18°C–19°C, light intensity of 30 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} with photoperiod of 16 h of light and 8 h of dark.

Microscopy. Cultures and plankton net samples were treated in order to remove the organic matter from diatom frustules using Simonsen's cleaning method (Simonsen 1974, Hasle 1978). The samples (~5 mL) were first rinsed with distilled water, followed by the addition of an equal amount of saturated $KMnO_4$ (or diluted 50%) and allowed to react for 24 h. The next day an equal amount of concentrated HCl was added, gently heated over an alcohol burner flame and then rinsed again with distilled water minimum five times until free of acid. Permanent slides were prepared by drying cleaned material on coverslips and mounting in Naphrax following Hasle (1978). Light microscopy observations were performed with a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with DIC and phase contrast and an Olympus BX51 microscope (Olympus). Permanent slides chosen for holotype material of new species are deposited at Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany while isotypes and original plankton net material from stations P150, P600, and P1000 are deposited at Macedonian National Diatom Collection (MKNDC) at Institute of Biology, Faculty of Natural Sciences, Skopje, Macedonia. Permanent slides of original collections (plankton net samples) are deposited at Department of Biology, Faculty of Science, University of Zagreb and available from authors (M.M. and S.B.) upon request.

For SEM, culture material was directly filtered on 3 μm pore-size Nucleopore polycarbonate membrane filters (Nucleopore, Pleasanton, CA, USA) and air-dried. Dehydration was done in the ethanol-series (25%, 35%, 50%, 75%, 80%, 90%) prepared with distilled water and absolute ethanol finishing with three rinses of 100% ethanol, 5 min at each step. For drying, a chemical agent, hexamethyldisilazane (HDMS) was used (Bray et al. 1993). The samples were rinsed in a series of 100% ethanol: HDMS solution (3:1, 1:1, 1:3), finishing with three rinses of 100% HDMS, 5 min treatment at each step, allowing the last HDMS rinse to evaporate slowly at room temperature. The filters were placed on aluminum stubs, coated with 15 nm gold using Scancoat Six Sputter Coater (BOC Edwards, Wilmington, MA, USA) and examined with a SEM FEG Tescan MIRA3 microscope (Brno, Czech Republic). For TEM, cleaned material was directly deposited onto Formvar-carbon coated copper grids, air-dried and examined with a FEI Morgagni 268D microscope (Eindhoven, the Netherlands).

The general diatom terminology used for the morphological descriptions follows Ross et al. (1979). Specific terminology for *Entomoneis* follows Paddock and Sims (1981), Osada and Kobayasi (1985) and Mejdandžić et al. (2017). The "junction line" introduced by Cleve (1894) has featured prominently in the taxonomy and classification of *Entomoneis* and related genera. This term has historically been applied to the area of the valve where the keel and valve body meet, which dependent on the thickness of the valve, the angle of the elevation of the keel, and the presence, position, and arrangement of basal fibulae, can attain a different shape (linear, sinusoid). In many cases the junction line can be difficult to accurately describe without SEM and TEM observations of fibulae. Although it has been suggested that all *Entomoneis* have a discernible junction line (Patrick and Reimer 1975), there are species that due to a variety of reasons (e.g., no basal fibulae; *E. aequabilis*), lack a junction line. Moreover, the junction line does not necessarily represent a homologous feature across species, because the impression of a junction line in the LM can result from thickening or overlap between structures, presence of basal fibulae, or some altogether different reason (i.e., angle at which a cell is observed). We therefore avoid using this term and whenever possible we describe the transition between keel and valve body through the constituent structures (that make up the impression of a junction line in the LM).

DNA isolation, PCR amplification, and sequencing. Genomic DNA was isolated from 50 mL of cell cultures obtained in exponential phase of growth using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purity of the extracted DNA was assessed with the NanoDrop spectrophotometer (BioSpec–nano; Shimadzu, Kyoto, Japan). The nuclear gene (18S rDNA) and two chloroplast-encoded genes (*rbc*L, *psb*C) were amplified using the EmeraldAmpMax PCR Master Mix© (Takara Bio, Kusatsu, Japan) following the PCR protocol described in Ruck and Theriot (2011). When necessary, nested PCR reaction was done with PCR product from first reaction as a template for second reaction. The primers used for amplification are listed in Table S1 in the Supporting Information. PCR products were visualized in a 1% agarose gel and then purified with StartaPrep PCR Purification Kit (Agilent Technologies, Inc. Santa Clara, CA, USA). The purified products were sent for Sanger sequencing (Macrogen© Europe, Amsterdam, The Netherlands, the Netherlands). All sequences were checked, edited and paired (5′–3′ and 3′–5′ ends) using Sequencher 4.1.4 (Gene Code Corporation, Ann Arbor, MI USA). Blast analysis was done for all sequences with blastn tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Multiple sequence alignment and phylogeny inference. A total of 48 taxa, 37 *Entomoneis* and 11 outgroup, were included in the phylogenetic analysis. Initial analyses included a broad set of outgroups from raphid diatoms including *Amphora* and *Halamphora*. However, the placement of *Entomoneis* within the Surirellales clade was strongly supported, so downstream analyses used outgroups from this clade and *Thalassiophysa*, which is consistently recovered as sister to the Surirellales (Stepanek and Kociolek 2014, Ruck et al. 2016). Data for *rbc*L and *psb*C were available for 48 and 42 accessions, respectively, while the SSU rDNA data set contained 44 accessions (Table 1). The two plastid genes, *rbc*L and *psb*C, were aligned based on their conceptual translations into amino acid sequences in Mesquite (Maddison and Maddison 2015). The SSU rDNA was aligned using Mafft v. 7.310 (Katoh and Standley 2013) accounting for the secondary structure of the RNA molecules. The alignments are available at: https://doi.org/10.5281/zenodo.804455.

We performed analyses on individual genes and on a concatenated three-gene alignment. In each case, we first identified the best model of nucleotide substitution and rate variation across sites using a model selection routine available in the program IQtree v. 1.5.5 (Nguyen et al. 2015). In addition, we performed a partition-merging procedure that joined two or more alignment partitions when the merge did not incur a substantial cost to the likelihood. Model and partition selection was done using the Bayesian information criterion (BIC) which penalizes for the number of parameters in a model and helps avoid overfitting. The initial partition models split the single-gene alignments into codons (where applicable), and the concatenated alignment was split first into genes and then into codons.

TABLE 1. List of 48 strains included in the phylogenetic analyses within this study with culture ID, location of strain isolation, and accession numbers for three different genes. Bold text used for new taxa or newly submitted sequences.

Taxon	Culture ID	Location	SSU	rbcL	psbC
Entomoneis tenera	PMFEN1	South Adriatic Sea, surface, Croatia		KX591887.1[a]	KX591884.1[a]
Entomoneis tenera	PMFEN2	South Adriatic Sea, surface, Croatia		KX591888.1[a]	KX591885.1[a]
Entomoneis tenera	PMFEN3	South Adriatic Sea, surface, Croatia	KX591890.1[a]	KX591889.1[a]	KX591886.1[a]
Entomoneis paludosa	L431	Cholla Lake, Arizona, USA	KX120688.1	KX120573.1	KX120458.1
Entomoneis paludosa var. *hyalina* = *Amphiprora hyalina*	CCAP1003/4	St. Agnesm, Cornwall, England, UK	KX120690.1	KX120575.1	KX120460.1
Entomoneis pulchra	L1853	Salton Sea, California, USA	KX120689.1	KX120574.1	KX120459.1
Entomoneis pulchra	CT10	Camp Theriot, Texas, USA	KX120694.1	KX120579.1	KX120464.1
Entomoneis pseudoduplex = *Amphiprora pseudoduplex*	ROS_AF18	Unknown	KP400266.1	KP400299.1	
Entomoneis pseudoduplex = *Amphiprora pseudoduplex*	ROS_AF18*	Unknown	KP400267.1	KP400300.1	
Entomoneis pseudoduplex = *Amphiprora pseudoduplex*	ROS_KD16	Ny Alesund, London, Kongsfjorden, Spitsbergen, Svalbard, sediment, Norway	KP400277.1	KP400303.1	
Entomoneis pseudoduplex = *Amphiprora pseudoduplex*	ROS_KD19	Unknown	KP400280.1	KP400304.1	
Entomoneis ornata	27D	Resthaven WMA, Ohio, USA	KX120693.1	KX120578.1	KX120463.1
Entomoneis ornata	14A	Paradise Lake, Michigan, USA	HQ912411.1	HQ912397.1	HQ912383.1
Entomoneis sp.	Roth5C	Gulf of Oman, Oman	KX120685.1	KX120570.1	KX120455.1
Entomoneis sp.	CC8	Clam Creek, Jekyll Island, Georgia, USA	KX120686.1	KX120571.1	KX120456.1
Entomoneis sp.	IK3b	Indian Keys, Florida, USA	KX120687.1	KX120572.1	KX120457.1
Entomoneis sp.	B3A	Belize	KX120691.1	KX120576.1	KX120461.1
Entomoneis sp.	CCMP467	California Bight, California, USA	KX120692.1	KX120577.1	KX120462.1
Entomoneis sp.	SA17B/2	Kariega River, South Africa	KX120695.1	KX120580.1	KX120465.1
Entomoneis sp.	3823/11	Matagorda Bay, Texas, USA	KX120696.1	KX120581.1	KX120466.1
Entomoneis sp.	3564/E1/1	Gab Gab, Guam	KX120697.1	KX120582.1	KX120467.1
Entomoneis sp.	CS782	Dunalley Bay, Tasmania, Australia	HQ912631.1	HQ912495.1	HQ912324.1
Entomoneis sp.	SZCZM496	Yantai coast, Yellow Sea, China	KT943630.1	KT943656.1	KT943689.1
Entomoneis sp.	MMOGRB 0374S	Fish culture pond in Fuqing, Fujian Province, China	KU525648.1	KX467260.1	
Entomoneis sp.	CCMP1693	Arabian Sea, Muscat, Oman	EF585586.1	JN162779.1	
Entomoneis infula **sp. nov. Mejdandžić & Bosak**	PMFT2ENT2	Middle Adriatic Sea, surface, Croatia	**MF000603.1**	**MF000628.1**	**MF000614.1**
Entomoneis umbratica **sp. nov. Mejdandžić & Bosak**	BIOTAII-21	South Adriatic Sea, 250 m, Croatia	**MF000604.1**	**MF000629.1**	**MF000615.1**
Entomoneis gracilis **sp. nov. Mejdandžić & Bosak**	BIOTAII-41	South Adriatic Sea, 30 m, Croatia	**MF000605.1**	**MF000630.1**	**MF000616.1**
Entomoneis adriatica **sp. nov. Mejdandžić & Bosak**	BIOTAII-49	South Adriatic Sea, 100 m	**MF000606.1**	**MF000632.1**	**MF000618.1**
Entomoneis gracilis **sp. nov. Mejdandžić & Bosak**	BIOTAII-60a	South Adriatic Sea, 100 m, Croatia	**MF000607.1**	**MF000633.1**	**MF000619.1**
Entomoneis infula **sp. nov. Mejdandžić & Bosak**	BIOTAII-68	South Adriatic Sea, surface, Croatia	**MF000608.1**	**MF000634.1**	**MF000620.1**
Entomoneis gracilis **sp. nov. Mejdandžić & Bosak**	BIOTAII-96	South Adriatic Sea, 30 m, Croatia	**MF000609.1**	**MF000635.1**	**MF000621.1**
Entomoneis cf. *alata*	BIOTAII-113	South Adriatic Sea, surface, Croatia	**MF000610.1**	**MF000636.1**	**MF000622.1**
Entomoneis vilicicii **sp. nov. Bosak & Mejdandžić**	PMFBION4A	South Adriatic Sea, surface, Croatia	**MF000611.1**	**MF000637.1**	**MF000623.1**
Entomoneis vilicicii **sp. nov. Bosak & Mejdandžić**	PMFBION4B	South Adriatic Sea, surface, Croatia		**MF000638.1**	**MF000624.1**
Entomoneis vilicicii **sp. nov. Bosak & Mejdandžić**	PMFBION4C	South Adriatic Sea, surface, Croatia		**MF000639.1**	**MF000625.1**
Entomoneis pusilla **sp. nov. Bosak & Mejdandžić**	PMFBIOP1	South Adriatic Sea, surface, Croatia	**MF000612.1**	**MF000640.1**	**MF000626.1**
Auricula mirabilis	4vi08/1cA		KX120682.1	KX120567.1	KX120453.1
Rhopalodia gibba	CYTX022		KX120675.1	KX120559.1	KX120447.1
Epithemia parallela	N09/43/rhop2/3		KX120676.1	KX120560.1	KX120448.1
Epithemia turgida	CYTX021		KX120679.1	KX120564.1	KX120451.1
Coronia daemeliana	3623/C/100		KX120707.1	KX120592.1	KX120476.1
Campylodiscus clypeus	LG3/CC		KX120700.1	KX120584.1	KX120470.1
Surirella brebissonii	KR1		KX120738.1	KX120620.1	KX120507.1

(continued)

TABLE 1. (continued)

Taxon	Culture ID	Location	SSU	rbcL	psbC
Surirella brightwellii	L1753		KX120730.1	KX120613.1	KX120499.1
Iconella curvula	L541		HQ912416.1	HQ912402.1	HQ912388.1
Iconella pumila	SwampA		KX120777.1	JX033016.1	KX120544.1
Thalassiophysa hyalina	4vi08/1cT		KX120669.1	KX120552.1	KX120440.1

[a] Correction of seven accession numbers belonging to sequences of each of three *E. tenera* strains published by Mejdandžić et al. (2017).

We reconstructed phylogenies using maximum likelihood (ML) and Bayesian inference (BI) in IQtree v. 1.5.5 (Nguyen et al. 2015) and MrBayes v. 3.2.6. (Ronquist et al. 2012), respectively. We performed 400 ML optimizations for each single-gene alignment and the concatenated three-gene matrix and chose the one with smallest BIC score as the "best tree". ML optimizations were performed under default settings in IQtree, each starting from a different random number seed, i.e. different point in parameter space, for a more exhaustive search of the likelihood surface. In addition, repeating the optimization many times, we varied the strength of perturbation of the nearest neighbor interchange during tree rearrangement, which is helpful for avoiding local optima during the likelihood optimization (Nguyen et al. 2015). Clade support was assessed using IQtree's UltraFast bootstrap routine (Minh et al. 2013) with 1,000 pseudoreplicates.

Bayesian analyses were carried out only for the concatenated alignment with the best set of partitions as identified by IQtree, but with different parametrization for the substitution rate matrix. Instead of the models identified as optimal by IQtree, we used a mixed model strategy, whereby various variants of the Generalized time-reversible model (GTR) were sampled in proportion to their posterior probability (MrBayes option "nst=mix"). Among-site rate variation in MrBayes was accommodated via a Γ distribution with four rate categories ($\Gamma 4$) and by estimating the proportion of invariant sites (I). We ran four simultaneous Markov chain Monte Carlo (MCMC) simulations, each composed of one cold and three heated chains for a total of 10 million generations with a sampling frequency of one thousand generations. Stationarity and convergence among the MCMC runs was assessed from the MrBayes output (standard deviation of split frequencies and potential scale reduction factor) and by inspecting the posterior distributions in the program Tracer v. 1.6 (Drummond and Rambaut 2007). The first 25% of the sampled posterior distributions were discarded as burn-in.

RESULTS

Phylogeny of the genus Entomoneis. Trees generated from single genes recovered *Entomoneis* as either monophyletic (*rbc*L) or paraphyletic (SSU rDNA and *psb*C) with either *Thalassiophysa*, or several outgroup taxa embedded within *Entomoneis* dependent on rooting (Figs. S1–S3 in the Supporting Information). Non-monophyly of *Entomoneis* was possibly due to the rather sparsely sampled outgroup with long branches (Figs. S1 and S2). However, the relevant nodes were not strongly supported and topological hypothesis tests did not reject the monophyly of *Entomoneis* for both *psb*C and SSU rDNA (Shimodaira-Hasegawa and Approximately Unbiased test, $P > 0.05$). Smaller, strongly supported clusters of *Entomoneis* taxa (e.g., *E. tenera*/*E. infula*/*E. adriatica*) were consistently recovered across genes trees, especially for strains from the Adriatic Sea, but the relationships between them varied to a degree from gene to gene (Figs. S1–S3).

Analyses of the concatenated data set reconstructed *Entomoneis* as monophyletic with strong support (Bayesian posterior probability [PP]/

Bootstrap proportion [BS], PP/BS = 1/100, Fig. 1, A and B). The Bayesian majority rule consensus tree and the best tree found by maximum likelihood differed only in the placement of *E. pseudoduplex* strains (Fig. 1, A and B). Additionally, the placement of *E. pseudoduplex* clade was poorly supported by both Bayesian posterior probability and the bootstrap results, possibly due to the short SSU sequences and missing *psb*C data for these strains.

Entomoneis pseudoduplex aside, *Entomoneis* taxa were reconstructed into two clades, one containing *E. paludosa*, *E. ornata*, *E. pulchra* and several unidentified taxa (PP/BS = 0.68/70) and another that contained all strains from the Adriatic along with several unidentified and geographically scattered strains (PP/BS = 0.97/81; Fig. 1). The strains from the Adriatic were monophyletic and grouped into three subclades: (i) *Entomoneis* cf. *alata* BIOTAII–113 strain and two strains from Kariega River, South Africa and Gab Gab, Guam; (ii) four newly sequenced Adriatic strains, *E. tenera*, and a species isolated from the Arabian Sea and (iii) the remaining Adriatic strains which formed strongly supported sister relationships with *Entomoneis* strains isolated from very distant locations ranging from California to Tasmania (Fig. 1, A and B; Table 1).

Based on the phylogenetic results and the morphological data presented below, we describe six new *Entomoneis* species (1–6; Fig. 1, A and B). On the ML phylogeny, all but one of these species were either monophyletic groups or single branches (Fig. 1, A and B).

The exception *Entomoneis gracilis* sp. nov. represented by strains BIOTAII–41, BIOTAII–60a, and BIOTAII–96 was paraphyletic with respect to a single strain corresponding to *Entomoneis pusilla* sp. nov. (PMFBIOP1; Fig. 1, A and B). However, as described above, the placement of these strains differs in respect to SSU gene, but more importantly, the two species exhibit completely different morphological features (i.e., general appearance of the frustules, valve shape, striae perforations, degree of silification, and ultrastructure of girdle bands) therefore we describe these as separate species.

The clade composed of *Entomoneis gracilis* and *E. pusilla* was sister to *Entomoneis* from Tasmania (CS782) and this entire clade was a strongly supported sister to a group of *Entomoneis vilicicii* sp. nov. (PMFBION4A, PMFBION4B, PMFBION4C) and an *Entomoneis* isolate from California (CCMP467). The sister group to this clade was a lineage composed of *E. tenera* and an additional three new species from the Adriatic (Fig. 1, A and B). The first split within this lineage was between *Entomoneis umbratica* sp. nov. (Adriatic Sea: BIOTAII–21, Arabian Sea: CCMP1693) and a clade composed of *Entomoneis adriatica* sp. nov. (BIOTAII–49), *Entomoneis infula* sp. nov. (BIOTAII–68, PMFT2EN2), and *E. tenera* (Fig. 1, A and B). The last remaining strain isolated from the Adriatic Sea, provisionally called

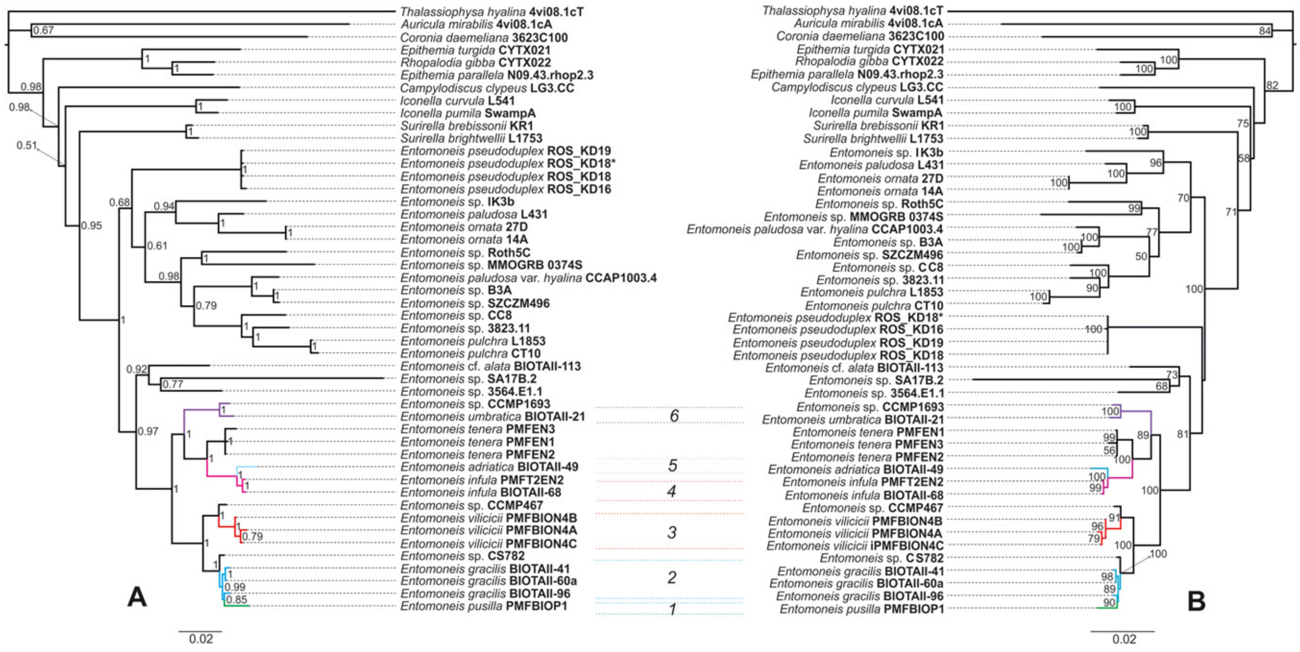

FIG. 1. (A) Majority rule phylogram of the postburning distributions of the four MrBayes runs inferred from concatenated dataset of three markers: SSU rDNA, *rbc*L and *psb*C. Branch support is summarized above branches as Bayesian posterior probability; values lower than 0.5 not shown. (B) "Best" Maximum Likelihood tree inferred from concatenated dataset of three markers: SSU rDNA, *rbc*L and *psb*C. Branch support is summarized above branches as Maximum Likelihood bootstrap values, lower than 50% not shown. Six new species of the Adriatic strains are shown as *1*, *2*, *3*, *4*, *5* and *6*. [Color figure can be viewed at wileyonlinelibrary.com]

Entomoneis cf. *alata*, was sister to unidentified *Entomoneis* from South Africa and Guam.

Sister to the lineage that contained all strains from the Adriatic Sea was a poorly supported group of *Entomoneis* from a variety of geographic locations. Here, a group of freshwater isolates of *E. ornata* and *Entomoneis paludosa*, along with a brackish isolate from Indian Keys, FL, USA, were sister to a number of largely unidentified taxa originating from marine or brackish habitats (Fig. 1, A and B). Notably, *E. paludosa* var. *hyalina* was reconstructed phylogenetically far from the nominate variety suggesting the need for reinvestigation of these strains based on type material.

Morphological observations and description of new taxa. All six new species are presented separately with etymology and type material information, characteristic morphological parameters observed under light and electron microscopy, and remarks about distinctive morphological characters. Detailed morphological features of the Adriatic *Entomoneis* compared with four other similar *Entomoneis* species are presented in Table S2 in the Supporting Information. Regarding morphometric data, cell length corresponds to the length measured at widest part of the cell (including keels) lying in girdle view, and valve length corresponds to length of the individual valve positioned in valve view. A summary of morphological characters for delineation of the new, previously described and unidentified *Entomoneis* from the Adriatic Sea is presented in Table 2.

***Entomoneis pusilla* sp. nov. Bosak & Mejdandžić**

Diagnostic features: Entomoneis pusilla is easily identified based on its small size (cells 9.7–14.1 μm long, 2.6–10.0 μm wide at constricted central part and 5.4–11.1 μm in widest part), being by far the smallest member of *Entomoneis.* Additional diagnostic features include the striae number (valve body striae 40–55 in 10 μm; keel striae 57–60 in 10 μm) and dash-like shaped perforations, hooked terminal raphe endings, narrow girdle bands ornamented with teardrop shaped areolae and silica thickenings in interareolar area, number of perforations in valvocopulae areolae (14–29 in abvalvar and 24–32 perforations in advalvar areolae).

Type: Strain PMFBIOP1 is designated as *Entomoneis pusilla* sp. nov. Holotype slide of the strain PMFBIOP1 deposited in The Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany as BRM ZU10/84 (holotype illustrated in Fig. 2A). Isotype slide deposited at Macedonian diatom collection, Skopje, Macedonia under accession number 011647/MKNDC. GenBank accessions: MF000612.1 (SSU), MF000640.1 (*rbc*L), MF000626.1 (*psb*C).

Type locality: Croatia: Adriatic Sea, south–eastern coast (P1000 station, 42°24′ N, 17°55′ E). Plankton net sample collected on March 10, 2016 by S. Bosak.

Etymology: From Latin adjective "*pusilla*" (miniature, tiny, very small) referring to the typical cell size.

Description: Cells very small, with heavily silicified frustules (Fig. 2, A and B). One plate-like plastid, cells do not twist around the apical axis (Fig. S4A in the Supporting Information). Frustules panduriform in girdle view, constricted in half of the frustule length (Fig. 2B). Cells 9.7–14.1 μm long, 2.6–10.0 μm wide at constricted central part and 5.4–11.1 μm in widest part ($n = 30$). Valves lanceolate (Fig. 2, A and D), 9.7–14.1 μm long, 2.3–3.8 μm wide in central part ($n = 21$). Scalpeliform valve apices (Fig. 2, C and D). Well-silicified sigmoid raphe-bearing keel distinct in both valve and girdle views (Fig. 2, A, B and D). The transition from the elevated keel to the valve body creates an impression of a straight line visible in cleaned frustules in LM (Fig. 2B). Valve striation becomes apparent in EM (Fig. 2, C–H). Virgae are straight, simple, and extending parallel through whole valve body, with few virgae shorter than the rest (Fig. 2D). Valve striae 40–55 in 10 μm. Keel narrow, weakly bilobate with short parallel virgae and striae fusing along the transition to the valve body giving them radial appearance, often looking decussate (Fig. 2, C and D). Keel striae 57–60 in 10 μm. The striae are closed by a hymen with very narrow dash-like perforations arranged in two parallel lines along the striae edges (Fig. 2, C and E–G). Arrangement and density of the perforations denser in striae on the keel than on the valve body, 64–75 in 1 μm near the keel margin and 49–50 in 1 μm near the valve margin (Fig. 2C). Series of basal fibulae born on each keel virga are present along the transition to the valve body, 4–5 basal fibulae per 1 μm. Basal fibulae sometimes interconnected with adjacent ones with transverse connections in shape of H or Y (Fig. 2C). Sigmoid raphe with simple linear central endings and slightly hooked terminal endings (Fig. 2, E–G). The raphe slit is located at the keel apex. The raphe canal is deep and separated from the valve by raphe fibulae, except in the central nodule that extends over area with four virgae (Fig. 2, E and F). Raphe fibulae 50–60 in 10 μm, sometimes fused between each other forming H or K shape (Fig. 2E). All girdle bands are straight throughout the whole length, never appear crossed in girdle view (Fig. 2B). Both valvocopulae and copulae are narrow and share similar ultrastructure with two rows of distinct teardrop shaped areolae, and with silica thickenings in interareolar area (Fig. 2H). Areolae density in copulae same as in valvocopulae, 40–45 in 10 μm. Areolae are occluded by lightly silicified hymenes perforated with narrow dash-like perforations. In valvocopula there are 14–29 and 24–32 perforations in abvalvar and advalvar areolae respectively (Fig. 2H).

Remarks: In natural material observed valves were slightly more silicified than in cultures with a very

TABLE 2. Morphological comparison of seven planktonic Adriatic *Entomoneis* species: *E. tenera*, *E. gracilis*, *E. pusilla*, *E. vilicicii*, *E. infula*, *E. adriatica*, and *E. umbratica* nd – not defined; np – not present.

	Entomoneis tenera	*Entomoneis gracilis*	*Entomoneis pusilla*	*Entomoneis vilicicii*	*Entomoneis infula*	*Entomoneis adriatica*	*Entomoneis umbratica*
Cell torsion	Various degree of torsion around the apical axis	Rarely torsioned around the apical axis	Never torsioned	Torsioned usually only in wing area (lifted wing) or for 180° around the apical axis	Twisted around the apical and transapical axis (folded shape)	Rarely torsioned around the apical axis,	Various degree of torsion around the apical axis
Valve length/valve width (μm)	11–22/3–7	13.2–36.0/2.2–10.0	9.7–14.1/2.3–3.8	15.8–38.4/3.6–9.1	16.8–27.8/4.9–7.2	11.3–40.4/4.9–8.0	7.2–27.0/5.2–8.6
Intermediate fibulae	np	np	np	np	Scattered over the keel surface, as dotted thickenings of the virgae or connecting two adjacent virgae	np	Connecting two adjacent virgae, forming a continuous, distinct line.
Shape of stria hymen perforations/no. in 1 μm near the keel margin/no. in 1 μm near the valve margin	Rectangular perforations/26–37/20–39	Elongated rectangular perforations/32–41/36–41	Dash – like perforations/64–75/49–50	Roundish to elliptical perforations/32–38/24–34	Roundish to elliptical perforations/31–35/26–32	Round to elliptical perforations/nd/nd	Roundish to elliptical perforations/24–29/24–32
Girdle appearance	Crossed (decussate)	Straight	Straight	Straight except a few crossed bands in the central part	Crossed (decussate)	Crossed (decussate)	Crossed (decussate)
Valvocopulae shape and ultrastructure	Wide, transverse striae peforated with elongated perforations	Wide, transverse striae peforated with elongated perforations	Narrow, two rows of distinct teardrop shaped areolae, silica thickenings in interareolar area	Clepsydriform shape, transverse striae peforated with elongated perforations	Wide, transverse striae peforated with elongated perforations	Wide, transverse striae peforated with elongated perforations	Wide, transverse striae peforated with elongated perforations
No. of valvocopule striae in 10 μm/no. of poroids in abvalvar striae/no. of poroids in advalvar striae	56–60/nd/nd	56–60/31–42/6–8	40–45/14–29/24–32	55–60/19–44/10–17	50–60/25–27/6–9	50–65/nd/nd	50–65/10–21/7–14

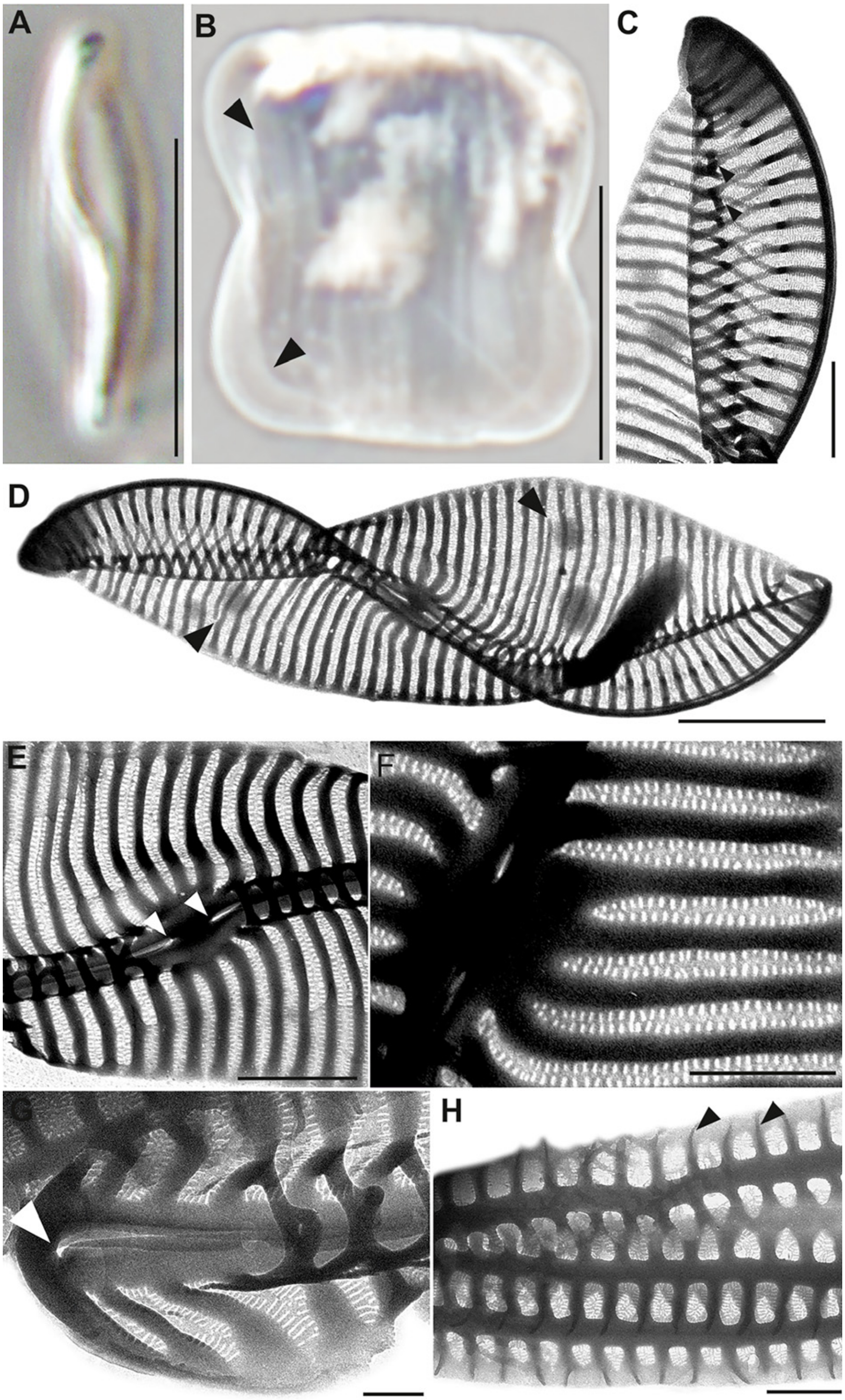

FIG. 2. *Entomoneis pusilla* sp. nov. Bosak & Mejdandžić (A–B) LM; (C–H) TEM; (A, C–H) strain PMFBIOP1; (B) natural material; (A) Lanceolate valve with distinct sigmoid raphe–bearing keel. (B) Panduriform cell in girdle view with distinct keel to valve body transition (arrowheads). (C) Terminal part of the valve with scalpeliform valve apex and the interconnected basal fibulae at the transition between keel and valve body (arrowheads). (D) Lanceolate valve with parallel virgae and striae on valve body and short virgae present (arrowheads). (E) Central part of the valve showing simple and straight central raphe endings (arrowheads). (F) Valve striation with fine dash-like perforations of the striae hymen. (G) Raphe canal with hooked terminal raphe ending. (H) Valvocopula and copula with teardrop shaped abvalvar and advalvar areolae and silica thickenings in interareolar area (arrowheads). Scale bars (A, B) 10 μm; (D) 2 μm; (C, E, and F) 1 μm; (H) 0.5 μm; (G) 0.2 μm. [Color figure can be viewed at wileyonlinelibrary.com]

distinctive keel (Fig. 2B). In prolonged culture conditions (after 6 months), no larger morphological abnormalities were observed, except the decrease in the constriction of the central part of the cell such that the bilobate shape of the keel was less pronounced.

In Mejdandžić et al. (2017) TEM micrographs published as figures 21, 22, 24, 26, and 31 and labeled as *Entomoneis tenera* actually depict specimens of *E. pusilla*.

***Entomoneis gracilis* sp. nov. Mejdandžić & Bosak**

Diagnostic features: Entomoneis gracilis can be delineated from other Adriatic *Entomoneis* based on its slender cell shape, number of striae perforations on the valve (32–41 in 1 μm near keel margin and 36–41 in 1 μm near the valve margin) together with a number of perforations within girdle band striae (31–42 poroids in abvalvar, 6–8 poroids in advalvar striae).

Type: Strain BIOTAII–60a, designated as *Entomoneis gracilis* sp. nov. Holotype slide of the strain BIOTAII–60a deposited in The Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany as BRM ZU10/85 (holotype illustrated in Fig. 3A). Isotype slide deposited at Macedonian diatom collection, Skopje, Macedonia under accession number 011650 MKNDC. GenBank accessions: MF000607.1 (SSU), MF000633.1 (*rbc*L), MF000619.1 (*psb*C).

Type locality: Croatia: Adriatic Sea, south-eastern coast (P1000, 42°20′ N, 17°49′ E). Plankton sample taken at 100 m depth, collected on March 10, 2016, by M. Mejdandžić.

Etymology: From Latin adjective "*gracilis*" (fine, narrow, slim, thin) referring to the general appearance and width of the cells.

Description: Cells of delicate appearance with lightly silicified frustules (Fig. 3, A and B; Fig. S4, B and C). One plate-like plastid (Fig. S4, B and C), cells often twisted around apical axis (Fig. 3C). Frustules panduriform in girdle view, constricted at half of the frustule length (Fig. 3, B, C and D). Cells 13.2–36.0 μm long, 3.8–17.6 μm wide at constricted central part and 5.5–21.4 μm in widest part (*n* = 50). Valves narrowly lanceolate, more lanceolate in smaller specimens while more linear in larger specimens (Fig. 3, A and E), 13.2–36.0 μm long, 2.2–10.2 μm wide (*n* = 46). Broad scalpeliform valve apices (Fig. 3E). Sigmoid raphe on an elevated keel distinct in valve view (Fig. 3, A and E). The transition from the elevated keel to the valve body creates an impression of a straight line, easily discernible in LM in larger specimens (Fig. 3B; Fig. S4C). Valve striation is not discernible in LM, but becomes apparent in EM (Fig. 3, F and G). Virgae are straight, parallel, and simple, extending through whole valve body, rarely bifurcated toward the valve margin. Sometimes, short virgae do not reach the valve margin but end at about half of the valve body (Fig. 3, E and F). Valve striae 35–45 in 10 μm. Bilobate keel narrow with parallel striae and virgae that fuse along the transition to the valve body giving them radial appearance (Fig. 3, E and F). Keel striae 40–52 in 10 μm. The striae are closed by a hymen with elongated rectangular perforations arranged in two parallel lines along the striae edges (Fig. 3, F and G). Arrangement and density of the perforations similar in striae on the keel and on the valve body (Fig. 3F), 32–41 in 1 μm near the keel margin and 36–41 in 1 μm near the valve margin. Series of basal fibulae are present along the transition to the valve body, 5–6 basal fibulae per 1 μm (Fig. 3H). Basal fibulae occasionally interconnected with adjacent fibulae with transverse connections in a H shape (Fig. 3E). Sigmoid raphe with simple and straight central and terminal endings (Fig. 3, G and I). The raphe slit is located at the apex of the keel (Fig. 3I). The raphe canal is separated from the valve by raphe fibulae which are often interconnected with transverse connections at one or even two levels, except in the central nodule that extends over area with four to six virgae (Fig. 3G). Raphe fibulae 42–55 in 10 μm. The cingulum is composed of one valvocopula and two to three copulae with smooth external surface (Fig. 3, D and H). All girdle bands are straight and do not appear crossed in girdle view (Fig. 3D). Both valvocopulae and copulae have similar ultrastructure with transverse striae occluded by very lightly silicified hymenes with elongated elliptical to rectangular perforations (Fig. 3, E and H), with 6–8 and 31–42 perforations in advalvar and abvalvar striae respectively (Fig. 3E). Striae density in copulae denser than in valvocopulae, 60–70 in 10 μm and 56–60 in 10 μm respectively. Abvalvar interstriae in valvocopulae often bifurcated and curled at the costa's end, mostly in larger specimens (Fig. 3J).

Remarks: In prolonged culture conditions slight morphological changes were observed in all three strains when compared to natural material and these were probably due to the cell size diminution. Observed changes in smaller cells included narrowing of the keel, valve shape becoming more lanceolate and less pronounced cell torsion.

***Entomoneis vilicicii* sp. nov. Bosak & Mejdandžić**

Diagnostic features: Entomoneis vilicicii can be discriminated from other *Entomoneis* by the clepsydriform shaped valvocopulae, unique cell torsion with only one wing positioned at 90° in respect to the rest of the cell and number of valvocopulae striae (55–60 in 10 μm) and within striae perforations (19–44 poroids in abvalvar, 10–17 poroids in advalvar striae).

Type: Strain PMFBION4A designated as *Entomoneis vilicicii* sp. nov. Holotype slide of the strain PMFBION4A deposited in The Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany as BRM ZU10/86 (holotype illustrated in Fig. 4, A–C). Isotype slide deposited at Macedonian diatom collection, Skopje, Macedonia under accession number

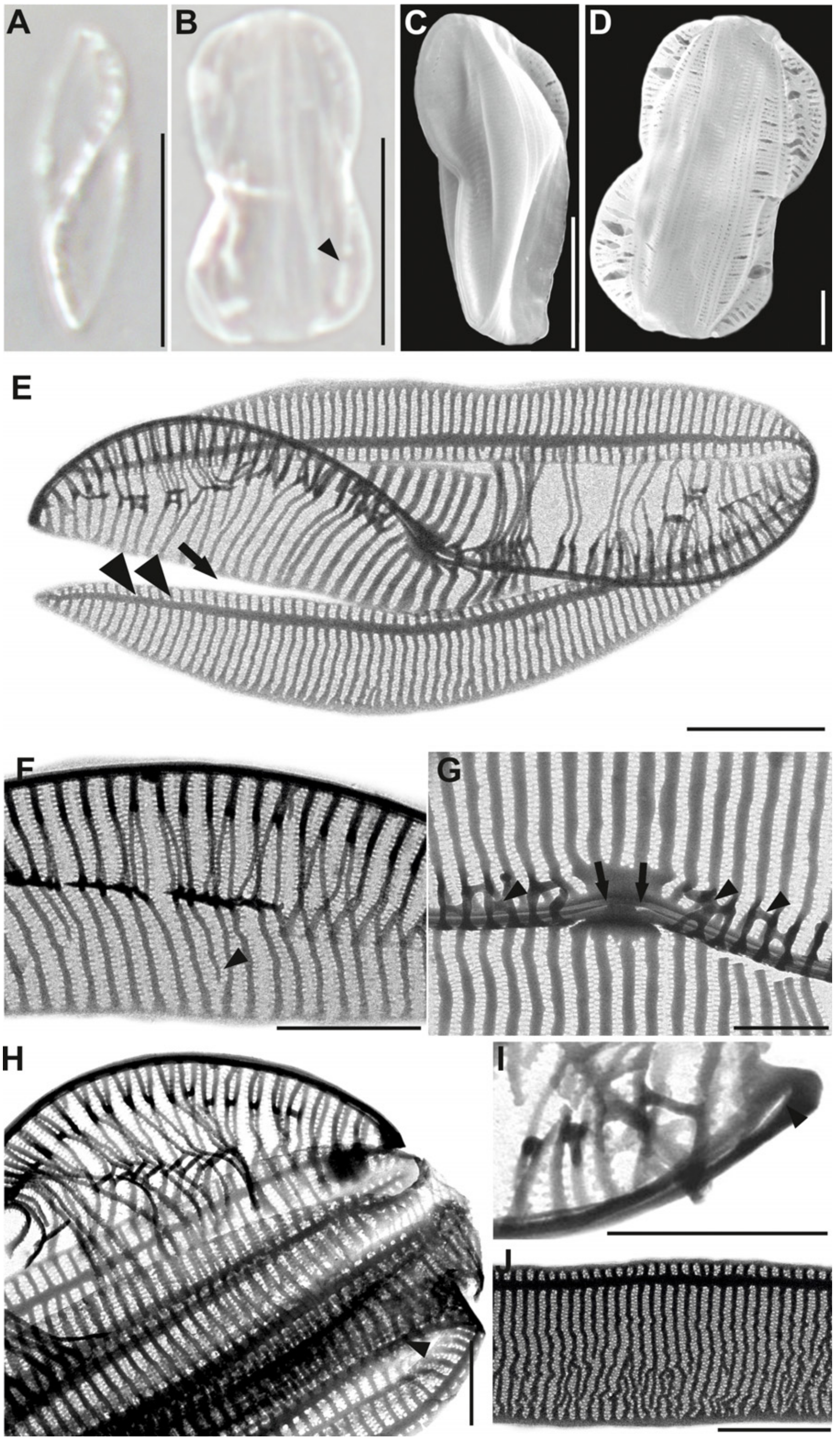

FIG. 3. *Entomoneis gracilis* sp. nov. Mejdandžić & Bosak (A, B) LM; (C, D) SEM; (E–J) TEM; (A, C, E–H) strain BIOTAII–60a; (D, I and J) strain BIOTAII–96; (B) natural material. (A) Linear-lanceolate valve with sigmoid raphe-bearing keel. (B) Frustule in girdle view with distinct basal fibulae at the transition between the keel and valve body (arrowhead). (C) Cell torsioned around the apical axis. (D) Frustule in girdle view showing smooth external surface of girdle bands. (E) Valve with attached valvocopula. Note the broad scalpeliform valve apices and valve striation with short virgae (arrow). Arrowheads point to interconnected basal fibulae. (F) Partial view of the valve showing striation with short valve virga (arrowhead). (G) Central part of the valve with simple and straight central raphe endings (arrows) and fused raphe fibulae (arrowheads). (H) Terminal part of the cell with simple and straight terminal raphe ending (arrowhead). (I) Close up of the broad scalpeliform apex with straight terminal raphe ending (arrowhead). (J) Wide valvocopula ornamented with bifurcated interstriae. Scale bars (A, B) 10 μm; (C) 5 μm; (D–F) 2 μm; (G–J) 1 μm. [Color figure can be viewed at wileyonlinelibrary.com]

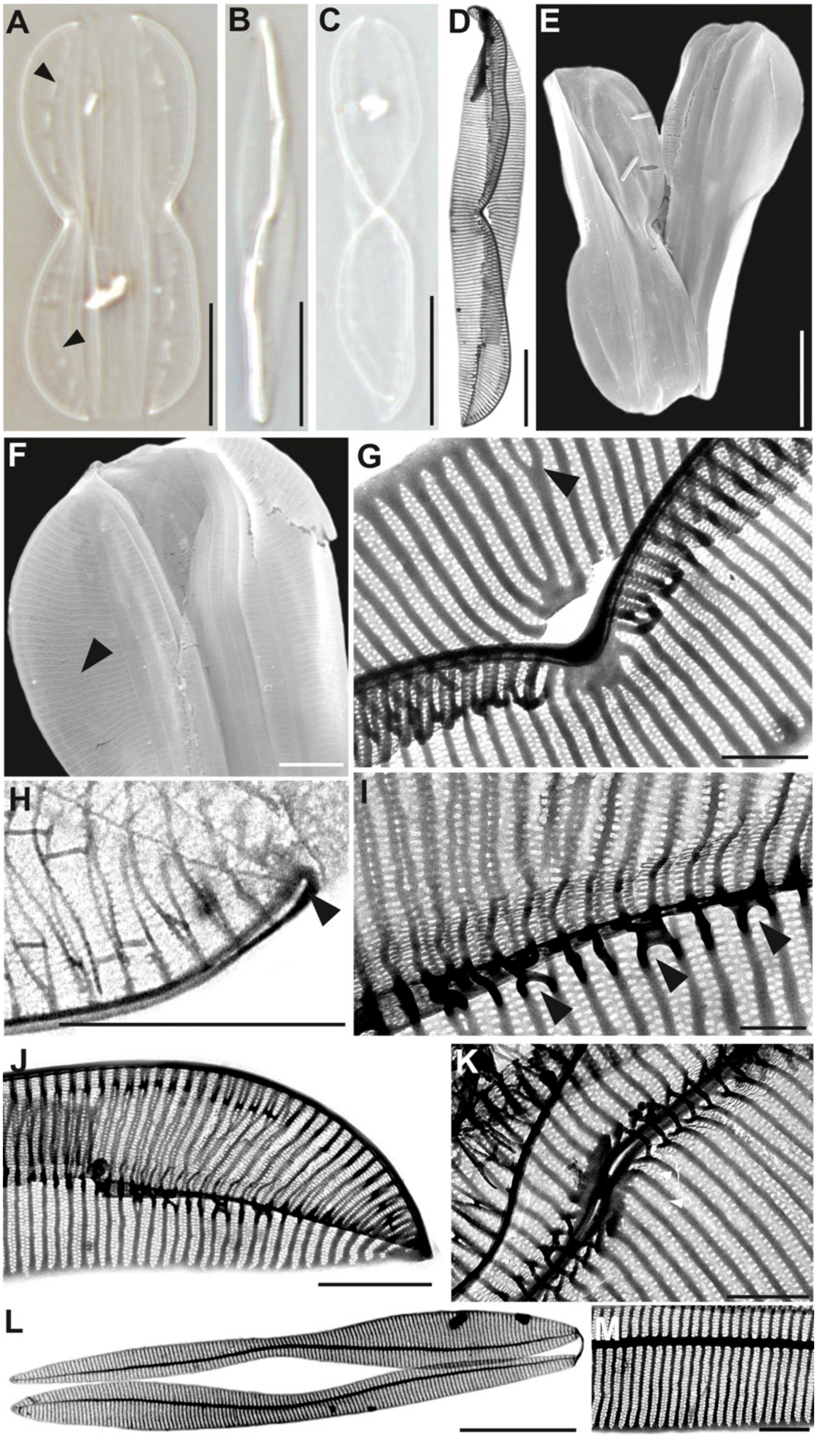

FIG. 4. *Entomoneis vilicicii* sp. nov. Bosak & Mejdandžić. (A–C) LM; (D, G–M) TEM; (E and F) SEM. (A–M) strain PMFBION4A. (A) Frustule in girdle view showing basal fibulae (arrowhead). (B) Linear-lanceolate valve with sigmoid raphe-bearing keel. (C) Cell twisted around the apical axis for 180°. (D) Linear-lanceolate valve with acute valve apices. (E) Two cells with characteristic torsion in only one wing being lifted for 90° in respect to the rest of the cell. (F) Terminal part of the cell with arrowhead pointing on keel virgae bifurcation. (G) Central part of the valve with arrowhead pointing on keel virgae bifurcation. (H) Valve apex with simple and straight terminal raphe end (arrowhead). (I) Basal fibulae with several interconnections (arrowhead) forming H shape. (J) Terminal part of the valve with acute valve apex and visible rows of basal and raphe fibulae. (K) Central portion of the valve showing straight central raphe endings (arrowheads). (L) Clepsydriform shaped valvocopulae. (M) Valvocopula ultrastructure. Scale bars (A–C, E) 10 μm; (L, D) 5 μm; (F, J) 2 μm; (H, G, J, K, M) 1 μm. [Color figure can be viewed at wileyonlinelibrary.com]

011648 MKNDC. GenBank accessions: MF000611.1 (SSU), MF000637.1 (*rbc*L), MF000623.1 (*psb*C).

Type locality: Croatia: Adriatic Sea, south-eastern coast (P600, 42°24′ N, 17°55′ E). Plankton net sample collected on March 10, 2016 by S. Bosak.

Etymology: The species has been named in honor to professor emeritus Damir Viličić, S.B. PhD supervisor and an outstanding scientist who considerably contributed to the knowledge on phytoplankton taxonomy and ecology in the Adriatic Sea.

Description: Large and delicate cells with lightly silicified frustules (Fig. 4, A–C; Fig. S4D). One plate-like plastid (Fig. S4D). Frustules panduriform in girdle view, constricted at half of the frustule length (Fig. 4, A and F; Fig. S4D). Cells commonly twisted only in wing area, appearing as one wing is torsioned (lifted) for 90° in respect to the rest of the cell (Fig. 4E), but also can sometimes be twisted up to 180° around the apical axis (Fig. 4C). Cells 15.8–38.4 μm long, 4.0–11.1 μm wide at constricted central part and 7.2–14.7 μm in widest part ($n = 81$). Valves narrowly lanceolate (Fig. 4, B and D), 15.8–38.4 μm long, 3.6–9.1 μm wide in central part ($n = 23$). Acute valve apices (Fig. 4, A and J). Well-silicified sigmoid raphe-bearing keel distinct in valve view (Fig. 4B). The transition from the keel to the valve body creates an impression of a straight to slightly arcuate line (Fig. 4, A, D and J; Fig. S4D). Valve striation becomes apparent in EM (Fig. 4, F–K). Virgae are straight, parallelly extending through the whole valve body, rarely bifurcated toward the valve margin (Fig. 4G). Valve striae 40–50 in 10 μm. Keel wider than the valve body, narrowly bilobate with parallel virgae and striae fusing along the transition to the valve body giving them radial appearance (Fig. 4, D, F and J). Keel striae 38–45 in 10 μm. Keel virgae sometimes bifurcated toward the keel apex (Fig. 4F). The striae are closed by a hymen with roundish to elliptical perforations arranged in two parallel lines along the striae edges (Fig. 4, G, H, J and K). Arrangement and density of the perforations denser in striae on the keel than on the valve body, 32–38 in 1 μm near the keel margin and 24–34 in 1 μm near the valve margin (Fig. 4J). Basal fibulae extending from keel virgae are present along the transition to the valve body, 4–6 basal fibulae in 1 μm. Basal fibulae sometimes interconnected with adjacent fibulae with transverse connections forming shape of letter H or Y (Fig. 4, I and J). Sigmoid raphe with simple linear central and terminal endings (Fig. 4, H and K). The raphe slit is located at the apex of the keel. The raphe canal is separated from the valve by raphe fibulae, except in the central nodule that extends over area with four to five virgae (Fig. 4, G and K). Raphe fibulae 40–50 in 10 μm. The cingulum is composed of one valvocopula and three copulae. Valvocopula has a characteristic clepsydriform shape, described as alternation of convex and concave band edge in respect to longitudinal rib (Fig. 4L). Valvocopulae and copulae share similar ultrastructure with transverse striae occluded by very lightly silicified hymenes with roundish to elliptical perforations (Fig. 4, L and M), with 10–17 and 19–44 perforations in advalvar and abvalvar valvocopula striae, respectively. Striae density in copulae similar as in valvocopulae, 50–60 in 10 μm and 55–60 in 10 μm respectively.

Remarks: No distinctive morphological abnormalities were observed in prolonged culture conditions in comparison to natural material (Fig. 4A). The changes in cell morphology included only features associated with size diminution (e.g., valves becoming more lanceolate and reduced cell torsion).

***Entomoneis infula* sp. nov. Mejdandžić & Bosak**

Diagnostic features: Entomoneis infula differs from other *Entomoneis* based on the general folded appearance of the cells, similar to a saddle shape. Other discriminating characters include decussate girdle bands, number of poroids within valvocopulae (25–27 poroids in abvalvar, 6–9 poroids in advalvar striae), and presence of intermediate fibulae irregularly scattered over the keel surface. Intermediate fibulae can be present as complete connections between virgae or only as dotted thickenings of the virgae, and are visible in cleaned frustules in LM.

Type: Strain BIOTAII–68 is designated as *Entomoneis infula* sp. nov. Holotype slide of the strain BIOTAII–68 deposited in The Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany as BRM ZU10/87 (holotype illustrated in Fig. 5A). Isotype slide deposited at Macedonian diatom collection, Skopje, Macedonia under accession number 011651 MKNDC. GenBank accessions: MF000608.1 (SSU), MF000634.1 (*rbc*L), MF000620.1 (*psb*C).

Type locality: Croatia: Adriatic Sea, south-eastern coast (P1000, 42°20′ N, 17°49′ E). Plankton net sample collected on March 10, 2016 by S. Bosak.

Etymology: The name is derived from Latin adjective "*infula*" (denoting a woollen fillet worn on the head by ancient Roman priests) referring to the general appearance of the cells.

Description: Delicate cells with lightly silicified frustules. One plate-like plastid, cells usually twisted around the transapical or apical axis appearing folded, or saddle shaped (Fig. S4E). Frustules panduriform in girdle view, constricted at half of the frustule length (Fig. 5E; Fig. S4E). Cells 16.8–27.8 μm long, 7.0–17.8 μm wide at constricted central part and 12.0–26.4 μm at widest part ($n = 31$). Valves linear-lanceolate (Fig. 5, A–C), 16.8–27.8 μm long, 4.9–7.2 μm wide at central part ($n = 31$). Scalpeliform valve apices (Fig. 5C). Well silicified sigmoid raphe-bearing keel distinct in valve view (Fig. 5A). Elevated keel transitions to the valve body creating an impression of an arcuate to slightly sinusoid line, well visible in LM (Fig. 5, A and B). Valve striation becomes apparent in EM (Fig. 5, C–H). Virgae are straight, parallelly extending through whole valve body toward the valve margin with some

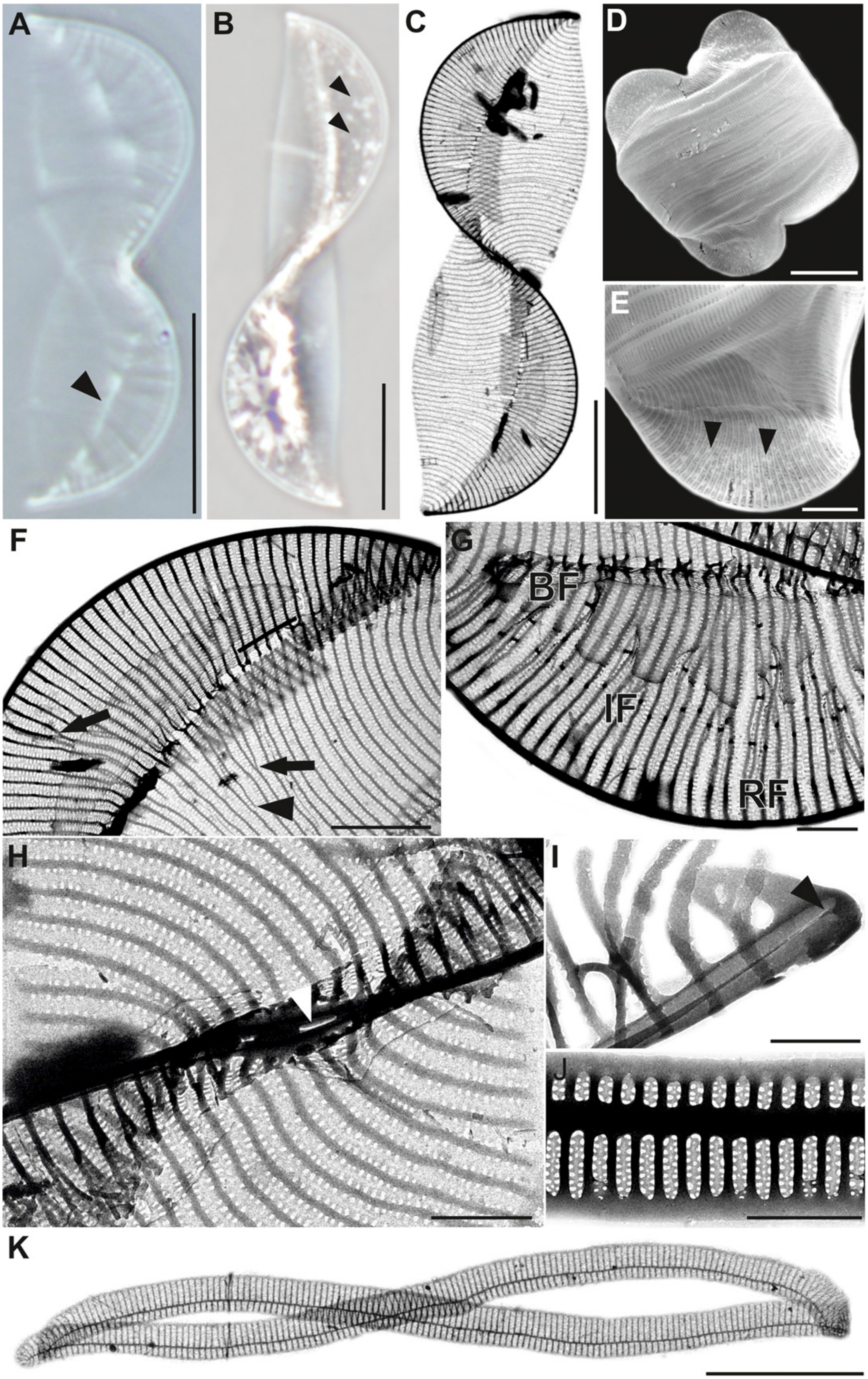
A
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FIG. 5. *Entomoneis infula* sp. nov. Mejdandžić & Bosak. (A–B) LM; (C, F–K) TEM; (D–E) SEM; (A, C–K) strain BIOTAII–68; (B) Natural material. (A) Valve in a girdle view with distinct basal fibulae (arrowhead). (B) Valve vith intermediate fibulae visible as small irregular dots on the keel (arrowhead). (C) Linear-lanceolate valve with sigmoid raphe-bearing keel and scalpeliform valve apices. (D) Panduriform cell showing cingulum with smooth external surface. (E) Detail of the valve body and keel with parallel valve striation, radial keel striation and scattered intermediate fibulae (arrowheads). (F) Detail of the valve body and keel showing bifurcated virgae (arrows) and short virgae insertions (arrowhead). (G) Valve body and keel with three types of fibulae: RF – raphe fibulae, IF – intermediate fibulae, BF – basal fibulae. (H) Central portion of the valve with straight central raphe endings (arrowhead). (I) Valve apex with straight terminal raphe end (arrowhead). (J) Valvocopula ultrastructure. (K) Valvocopula. Scale bars (A–C) 10 μm; (D, K) 5 μm; (E, F) 2 μm; (G, H, I, J) 1 μm. [Color figure can be viewed at wileyonlinelibrary.com]

of them not reaching the margin but finish at the middle part of the valve body (Fig. 5F). Virgae occasionally bifurcated toward the keel–body transition (Fig. 5F). Valve striae 35–50 in 10 μm. Keel wide, strongly bilobate with parallel virgae and striae fusing along the keel–body transition area giving them radial appearance (Fig. 5, C–F). Keel striae 40–55 in 10 μm. The striae are closed by a hymen with roundish to elliptical perforations arranged in two parallel lines along the stria edges (Fig. 5, F–H). Arrangement and density of the perforations denser in striae on the keel than on the valve body, 31–35 in 1 μm near the keel margin and 26–32 in 1 μm near the valve margin (Fig. 5, F and G). Series of basal fibulae born on each keel virga are present along the keel–body transition, 4–5 basal fibulae in 1 μm. Basal fibulae sometimes interconnected with adjacent ones with transverse connections forming H or Y shape (Fig. 5G). Intermediate fibulae connecting two neighboring keel virgae or dotted thickening of the virgae scattered over the keel surface between basal and raphe fibulae, 4–6 in 1 μm (Fig. 5, B, C and G). Sigmoid raphe with simple linear central and terminal endings (Fig. 5, H and I). The raphe slit is located at the apex of the keel (Fig. 5I). The raphe canal is shallow and separated from the valve by raphe fibulae, except in the central nodule that extends over surface area with three virgae on the valve body (Fig. 5H). Raphe fibulae 38–40 in 10 μm. The cingulum is composed of one valvocopula and three to four copulae with smooth external surface (Fig. 5D) and similar ultrastructure (Fig. 5, J and K). All girdle bands are crossed (Fig. 5K). Stria density in copulae same as in valvocopulae, 50–60 in 10 μm. Transverse striae occluded by very lightly silicified hymenes perforated with round to elliptical poroids, with 6–9 advalvar and 25–27 abvalvar poroids in valvocopulae striae respectively (Fig. 5J).

Remarks: No distinctive morphological abnormalities, including the preservation of the cell torsion that is in other species related to the decrease in cell size, were observed in prolonged culture conditions in comparison to natural material (Fig. 4B).

Entomoneis adriatica **sp. nov. Mejdandžić & Bosak**

Diagnostic features: Entomoneis adriatica is morphologically very similar to *E. gracilis*, however, the two species differ in the general appearance of the cells with distinct slender shape in the latter species. Other similar species is *E. tenera*, but its cells have crossed (decussate) girdle appearance while it is straight in *E. adriatica*.

Type: Strain BIOTAII–49 is designated as *Entomoneis adriatica* sp. nov. Holotype slide of the strain BIOTAII–49 deposited in The Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany as BRM ZU10/88 (holotype illustrated in Fig. 6B). Isotype slide deposited at Macedonian diatom collection, Skopje, Macedonia under accession number 011646 MKNDC. GenBank accessions: MF000606.1 (SSU), MF000632.1 (*rbc*L), MF000618.1 (*psb*C).

Type locality: Croatia: Adriatic Sea, south-eastern coast (P150, 42°32′ N, 17°59′ E). Plankton sample taken at 100 m depth, collected on March 8, 2016 by M. Mejdandžić.

Etymology: The species has been named after the Adriatic Sea, the area where the species has been discovered.

Description: Cells delicate with lightly silicified frustules. One multilobed plate plastid (Fig. S4, F and G). Cells usually torsioned around apical axis in various degrees (Fig. S4, F and G). Frustules panduriform in girdle view, constricted at half of the frustule length (Figs. 6C, S4F). Cells 11.3–40.4 μm long, 5.5–17.6 μm wide at constricted central part and 8.2–20.1 μm in widest part ($n = 20$). Valves linear-lanceolate (Fig. 6, A, C and D), 11.3–40.4 μm long, 4.9–8.0 μm wide at central part ($n = 30$). Scalpeliform valve apices (Fig. 6, A and B). Well-silicified sigmoid raphe-bearing keel distinct in valve view (Fig. 6, A and D). Transition from the elevated keel to the valve body creates an impression of an arcuate line, easily discernible in LM (Fig. 6, A and B). Valve striation becomes apparent in EM (Fig. 6, D, F, and H). Virgae are straight, parallel, and simple, extending through whole valve body (Fig. 6H). Valve striae 40–60 in 10 μm. Keel narrow, bilobate with parallel virgae and striae fusing along the keel–body transition giving them radial appearance (Fig. 6, C and I). Keel striae 45–50 in 10 μm. The striae are closed by a hymen with round to elliptical perforations arranged in two parallel lines along the striae edges (Fig. 6F). Series of 4–5 basal fibulae in 1 μm present along the keel–body transition (Fig. 6, C and I). Sigmoid raphe with simple and straight central and slightly downward curved terminal endings (Fig. 6, F, E, and G). The raphe slit is located at the apex of the keel. The raphe canal is separated from the valve by raphe fibulae, except in the central nodule that extends over the area with four virgae (Fig. 6E). Raphe fibulae 40–50 in 10 μm. The

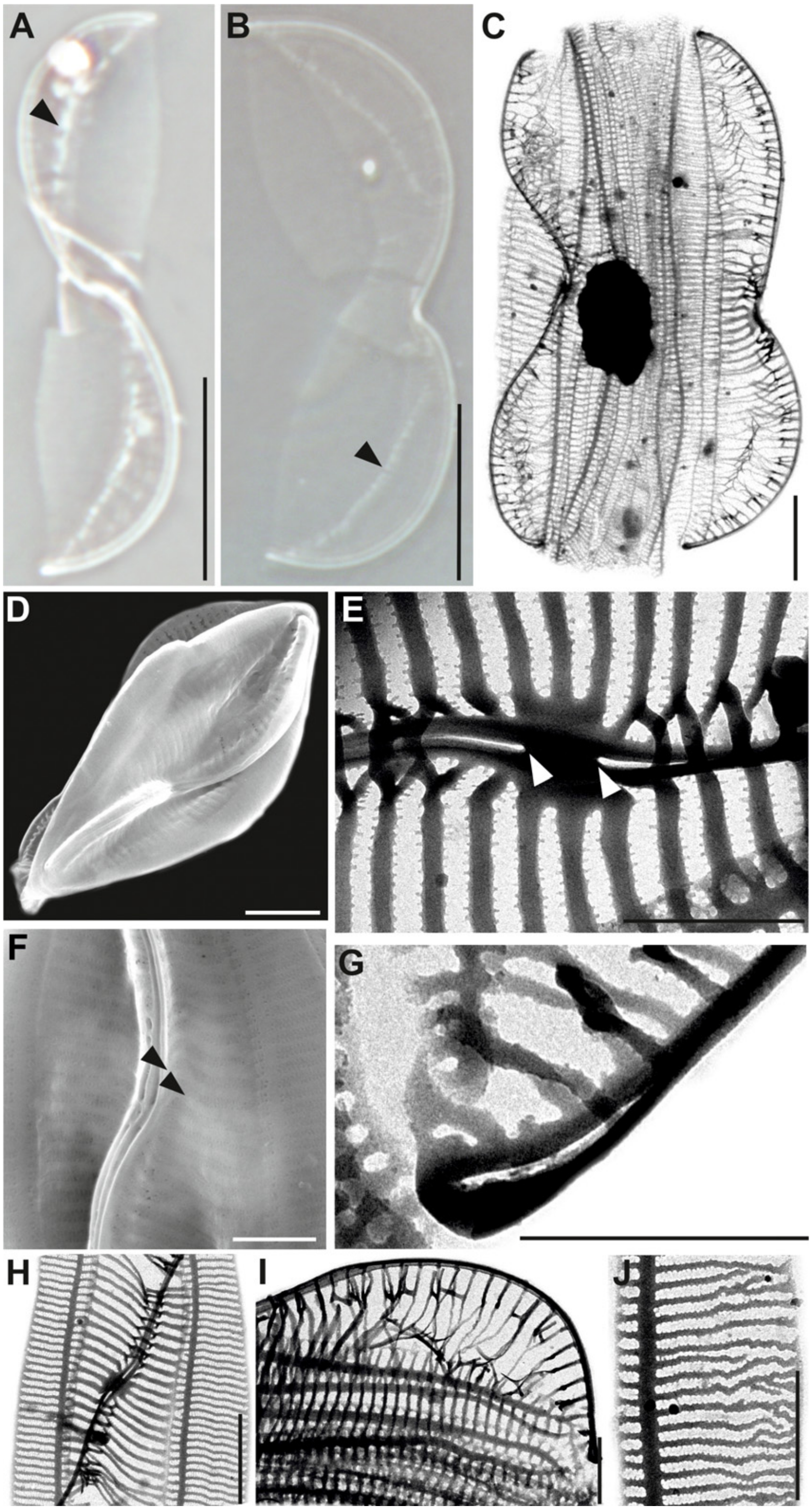

FIG. 6. *Entomoneis adriatica* sp. nov. Mejdandžić & Bosak. (A, B) LM; (C, E, G–J) TEM; (D, F) SEM. (B–J) strain BIOTAII–49; (A) Natural material (A) Valve in valve view with acute valve apices and distinct basal fibulae (arrowheads). (B) Valve in girdle view with an arcuate transition between the keel and valve body formed by basal fibulae (arrowheads). (C) Frustule in girdle view with straight appearance of the girdle. (D) Twisted cell. (E) Central part of the valve with straight central raphe endings (arrowheads). (F) Double raphe canal (arrowheads) in malformed cell in a prolonged culture. (G) Acute valve apex with straight terminal raphe end. (H) Central part of the valve with attached valvocopula. (I) Terminal part of the cell with distinct basal fibulae forming transition between the keel and valve body. (J) Detail of a valvocopula with bifurcated striae. Scale bars: (A, B) 10 μm; (C) 5 μm; (D, H) 2 μm; (E–G, I, J) 1 μm. [Color figure can be viewed at wileyonlinelibrary.com]

cingulum is composed of one valvocopulae and three copulae (Fig. 6I). All girdle bands are straight and do not appear crossed in girdle view (Fig. 6C). Valvocopulae and copulae have similar ultrastructure with transverse striae occluded by very lightly silicified hymenes perforated with round to elliptical poroids (Fig. 6, H and J). Abvalvar interstriae in valvocopula sometimes with bifurcated and curled ends (Fig. 6J). Striae density in copulae same as in valvocopulae, 50–65 in 10 μm.

Remarks: In prolonged cultured period, some specimens developed a double raphe canal as seen in Figure 6F – another interrupted slit in the raphe canal near the original raphe is visible. Additionally, natural shape variation occurred due to decrease in cell size with valves becoming more lanceolate shaped and cells less twisted.

***Entomoneis umbratica* sp. nov. Mejdandžić & Bosak**

Diagnostic features: Entomoneis umbratica cells have conspicuously bilobate keel that is markedly constricted in the middle part of the valve, distinctive intermediate fibulae arranged in a form of an irregular, continuous line visible in LM and the number of perforations within striae on the valve (24–29 in 1 μm near keel margin and 24–32 in 1 μm near the valve margin) and valvocopulae (10–21 poroids in abvalvar, 7–14 poroids in advalvar striae).

Type: Strain BIOTAII–21 is designated as *Entomoneis umbratica* sp. nov. Holotype slide of the strain BIOTAII–21 deposited in The Friedrich Hustedt Diatom Study Centre, Bremerhaven, Germany as BRM ZU10/89 (holotype illustrated in Fig. 7, A and C). Isotype slide deposited at Macedonian diatom collection, Skopje, Macedonia under accession number 011649 MKNDC. GenBank accessions: MF000604.1 (SSU), MF000629.1 (*rbc*L), MF000615.1 (*psb*C).

Type locality: Croatia: Adriatic Sea, south-eastern coast (42°24′ N, 17°55′ E). Plankton sample taken at 250 m depth, collected on March 10, 2016 by M. Mejdandžić.

Etymology: The name is derived from Latin adjective "*umbratica*" which means living in shade, devoted to sheltered leisure. Refers to the low light conditions in the water column at 250 m, the source of original material from which the species has been isolated.

Description: Delicate cells with lightly silicified frustules. One multilobed plate plastid (Fig. S4H). Cells variously torsioned around the apical axis (Fig. S4H; Fig. 7C). Frustules panduriform in girdle view, markedly constricted at half of the frustule length (Fig. 7A). Cells 7.2–27.0 μm long, 5.9–14.7 μm wide at constricted central part and 12.4–22.0 μm at widest part ($n = 25$). Valves linear-lanceolate (Fig. 7B), 7.2–27.0 μm long, and 5.2–8.6 μm wide at central part ($n = 25$). Acute valve apices (Fig. 7D). Sigmoid raphe-bearing keel well silicified and distinct in valve view (Fig. 7B). Elevated keel transitions into the valve body creating an impression of an arcuate line, easily discernible in LM (Fig. 7, A–C). Valve striation becomes apparent in EM (Fig. 7, D–H). Virgae are straight, parallel, and simple, extending through whole valve body, rarely bifurcated toward the keel–body transition (Fig. 7G). Valve striae 40–55 in 10 μm. Wide keel markedly bilobate with parallel virgae and striae fusing along the keel–body transition giving them radial appearance (Fig. 7D). Keel striae 40–55 in 10 μm. The striae are closed by a hymen with roundish to elliptical perforations arranged in two parallel lines along the stria edges (Fig. 7, E and H). Series of 4–5 basal fibulae in 1 μm born on each keel virga along the keel–body transition area. Basal fibulae sometimes interconnected with adjacent ones with transverse connections forming H or W shape (Fig. 7E). Intermediate fibulae interconnecting two adjacent keel virgae characteristically arranged in a form of an irregular, continuous line, 4–6 intermediate fibulae in 1 μm (Fig. 7, B and F). Sigmoid raphe with simple and straight central and terminal endings (Fig. 7, H and I). The raphe slit is located at the keel apex. The raphe canal is separated from the valve by raphe fibulae, except in the central nodule that extends over area with three to five virgae (Fig. 7H). Raphe fibulae 40–45 in 10 μm. The cingulum is composed of one valvocopulae and three copulae with smooth external surface (Fig. 7, G and J). All girdle bands cross each other, appearing decussate (Fig. 7J). Copulae and valvocopulae have similar ultrastructure with 55–70 transverse striae in 10 μm and 50–65 striae in 10 μm respectively. Striae are occluded by very lightly silicified hymenes perforated with round to elliptical poroids with 7–14 advalvar and 10–21 abvalvar poroids in valvocopulae striae respectively (Fig. 7K).

Remarks: In prolonged culture conditions, cell morphology changes due to the decrease in size and the panduriform shape is not always obvious as in the original material.

DISCUSSION

The vast diversity of raphid diatoms is most commonly associated with benthic habitats where their actin+myosin powered (Poulsen et al. 1999) and substrate-dependent motility mechanism should be most beneficial (Consalvey et al. 2004, Cohn et al. 2015). Nevertheless, there are lineages of raphid diatoms that have made evolutionary transitions into the planktonic realm, most notably the diverse *Fragillariopsis*+*Pseudo–nitzschia* clade (Bacillariales; Kooistra et al. 2007) as well as species of *Nitzschia, Cylindrotheca, Haslea,* and *Pleurosigma* (Hasle and Syvertsen 1997, Malviya et al. 2016). Other instances of raphid diatoms that have made similar benthos–to–plankton transitions (e.g., *Meuniera* and *Entomoneis*) are comparatively far less known (Hasle and Syvertsen 1997, Ashworth et al. 2017). *Entomoneis* is only distantly related to the previously mentioned

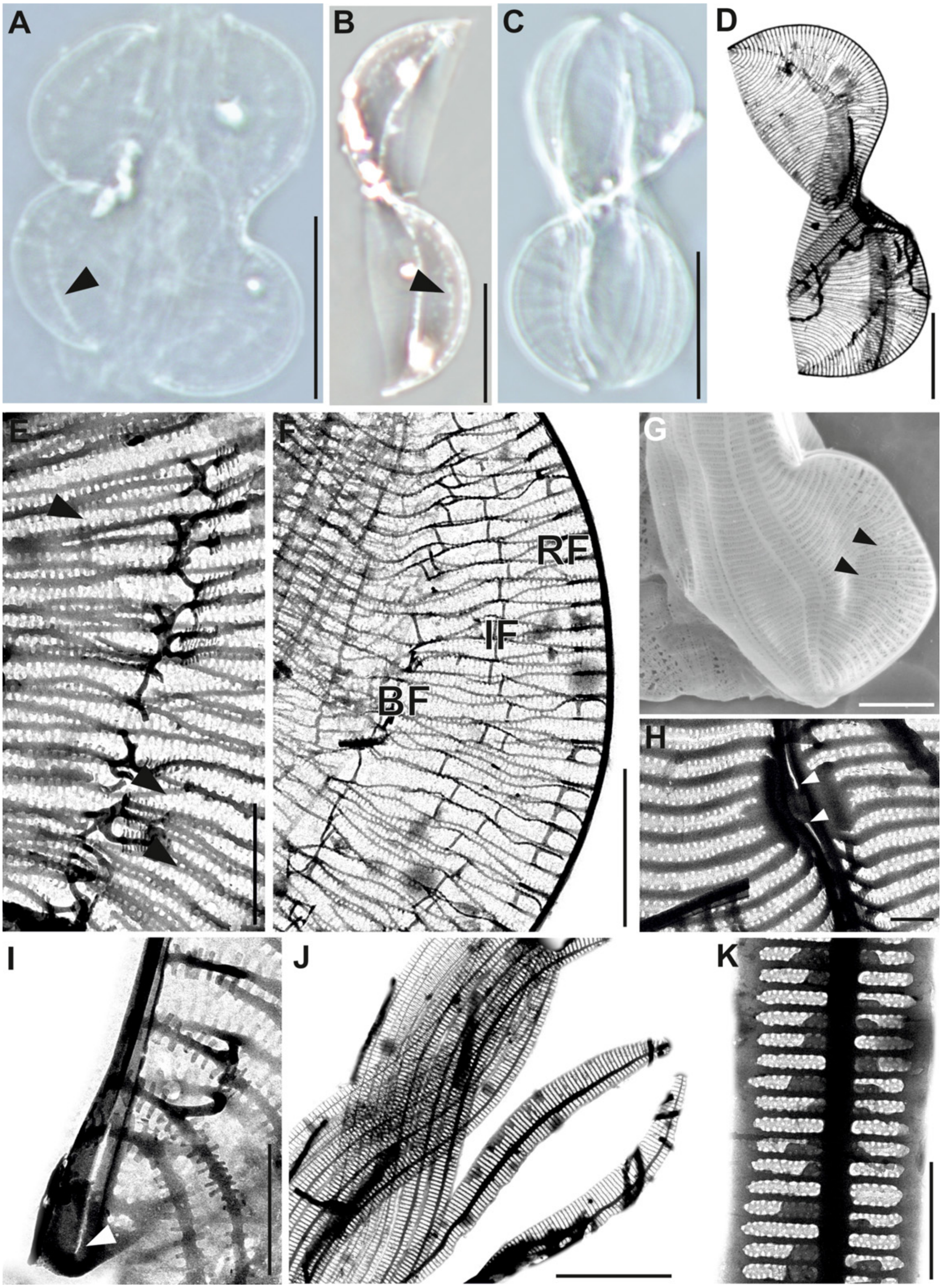

Fig. 7. *Entomoneis umbratica* sp. nov. Mejdandžić & Bosak. (A–C) LM; (D–F, H–K) TEM; (G) SEM. (A, C–K) strain BIOTAII–21; (B) natural material. (A) Cell in girdle view with markedly bilobate keels and an arcuate transition between the keel and valve body (arrowhead). (B) Linear-lanceolate valve with sigmoid raphe-bearing keel and distinct row of intermediate fibulae (arrowhead). (C) Cell torsioned for 180° around apical axis. (D) Valve with acute valve apices. (E) Detail of the valve with interconnected basal fibulae (arrowheads). (F) Partial view of the keel with RF – raphe fibulae, IF – intermediate fibulae and BF – basal fibulae. (G) Partial view of the frustule showing bifurcations of keel virgae (arrowheads) and smooth external surface of the cingulum. (H) Central part of the valve with straight central raphe endings. (I) Valve apex with straight terminal raphe end. (J) Crossed (deccusate) girdle bands. (K) Detail of valvocopula. Scale bars: (A–C) 10 µm; (D) 5 µm; (E–G, J) 2 µm; (H, I, K) 0.5 µm. [Color figure can be viewed at wileyonlinelibrary.com]

and belongs to a clade of otherwise nearly exclusively benthic species (Surirellales), so it is likely that *Entomoneis* evolved planktonic lifestyle independently of the other planktonic raphid pennates. In this study, we focused on the planktonic diversity in the genus *Entomoneis* (Surirellales), a common floristic component of marine, brackish and, to a lesser degree, freshwater habitats, with largely unknown phylogenetic and ecological history.

While it is not uncommon to observe *Entomoneis* in near-shore plankton tows, where cells from the benthos can get entrained in the water column by wave or tidal action (tychoplankton), off-shore planktonic *Entomoneis* are reported far less frequently, and are perhaps best known from polar habitats (Sutherland 2008, Poulin et al. 2011). *Entomoneis* from deep-water, temperate or tropical plankton are virtually unexplored, and when observed, commonly remain unclassified to the species level (Harnstrom et al. 2009, Armbrecht et al. 2015). To a degree, the poor understanding of species diversity in *Entomoneis* is a result of a lack of taxonomic and phylogenetic framework against which newly recorded specimens, especially from the plankton, can be compared. On one side, the generally complex three-dimensional structure and light silification of planktonic *Entomoneis* frustules requires detailed light and electron microscope observations of the valve and girdle elements for accurate species identification. On the other, molecular data for phylogeny reconstruction of *Entomoneis* are scattered across several studies (Ruck and Theriot 2011, Sorhannus and Fox 2012, Ruck et al. 2016, Dabek et al. 2017), are frequently without reliable species-level identification (Lundholm et al. 2002, Witkowski et al. 2016, Ashworth et al. 2017), and have not been analyzed together. A better understanding of the taxonomy, diversity, and ecology of this genus would benefit from a synthesis of the available morphological and phylogenetic data. In this study, we partially addressed some of these problems through a comparative investigation of fine-scale ultrastructure of several, mainly planktonic *Entomoneis*, and by combining all previously sequenced and new species of *Entomoneis* in a common phylogenetic analysis.

Morphological diversity of Adriatic Entomoneis. The new data presented in this study originate from samples collected from the central and south-eastern parts of the Adriatic Sea. Although investigating a relatively small geographic area, we found that *Entomoneis* in the Adriatic Sea represents a morphologically diverse assemblage of generally closely related species, six of which, *E. pusilla*, *E. gracilis*, *E. vilicicii*, *E. infula*, *E. adriatica,* and *E. umbratica*, we here described as new. The major differences between Adriatic *Entomoneis* were in cell shape, the degree and mode of torsion, shape of valve apices, presence and structure of intermediate fibulae, the ultrastructure and the general appearance of the girdle bands, and the arrangement and density of perforations along the valve and valvocopulae (Table 2, Table S2).

Although closely related (Fig. 1, A and B, clades *1* and *2*), *Entomoneis pusilla* and *Entomoneis gracilis* differ substantially in their morphology (Figs. 2 and 3; Table 2). *Entomoneis pusilla* cells are very small, with the upper size limit barely overlapping with *E. gracilis* lower size limit, 14.1 μm and 13.2 μm respectively. Another difference is the hooked terminal raphe endings in *E. pusilla* that are straight in *E. gracilis.* Ultrastructure of valve striae is markedly different as well, with *E. pusilla* having very distinctive narrow dash-like hymen perforations and on the other hand *E. gracillis* has striae hymen perforated with larger round to elliptical perforations. The same shape of hymen perforations is found in girdle bands of both species, *E. pusilla* has very narrow girdle bands ornamented with distinctive teardrop shaped areolae, while in *E. gracilis*, the girdle bands are much wider and ornamented with transverse striae.

The three strains grouped in Clade *3* (Fig. 1, A and B), represent *Entomoneis vilicicii*, one of the largest *Entomoneis* species found in the Adriatic Sea (15.8–38.4 μm long, 4.0–11.1 μm wide at constricted central part and 7.2–14.7 μm including wings). The largest Adriatic planktonic *Entomoneis* recorded so far, *E. adriatica* (clade *5*) has cells 11.3–40.4 μm long, 5.5–17.6 μm wide at constricted central part and 8.2–20.1 μm in widest part. Although comparable in size to many non-Adriatic *Entomoneis*, both species have unique morphological characters allowing straightforward identification. *Entomoneis vilicicii* cells usually have a unique mode of cell torsion, with only one lobe of the keel twisted by 90°, that has not been found previously among the members of this genus, although some smaller specimens were observed with the usual cell torsion (Fig. 4D). Moreover, *E. vilicicii* has unique clepsydriform shape of valvocopulae and highest density of poroids in advalvar and abvalvar transverse striae (Fig. 4M). *Entomoneis adriatica*, on the other hand, has a more usual mode of cell torsion (in various degrees around apical axis) and its valvocopulae are not clepsydriform. Similar to *E. tenera*, *E. adriatica* also has multilobed plate plastid (Fig. 6, A and B). In addition to cell size, differences between *E. tenera* and *E. adriatica* also include girdle appearance (*E. adriatica* straight, *E. tenera* decussate), striae density (denser in *E. adriatica*), perforations densities (denser in *E. tenera*), valve shape (*E. tenera* has broad lanceolate while *E. adriatica* has linear-lanceolate valves) and form of valve apices (broad scalpeliform in *E. tenera* and acute in *E. adriatica*).

Entomoneis infula, represented by two strains in Clade *4* (Fig. 5, A–L), is easily identified under LM due to its characteristic folded (saddle-shaped) cells. This type of cell torsion along the transapical, rather than apical axis has not been documented for *Entomoneis* before and is more typical for some other canal-raphe diatom genera like *Iconella*, *Surirella,* and *Campylodiscus* (Ruck et al. 2016). Among the other morphological characters, the keel–body transition in *E. infula* has unique shape, intermediate between arcuate as in *E. punctulata*, and sinusoid as in *E. paludosa* (Osada and Kobayasi 1990c). Additionally, *E. infula* has intermediate fibulae present between the keel and the basal fibulae. This feature is shared with *E. japonica*, *E. pseudoduplex*, and *E. umbratica*, but unlike the single-row intermediate fibulae found in these species, the intermediate fibulae of *E. infula* are irregularly scattered on the keel surface (Fig. 1, clade *6*; Osada and Kobayasi 1985, 1990c). Although *E. infula* is sister to *E. tenera*, these two species are different in many respects. *Entomoneis tenera* has multilobed plastid, straight to slightly arcuate impression of the keel–body transition, broad lanceolate valves, exclusively apical torsion, rectangular perforations in striae, while *E. infula* has plate-like plastid, arcuate to sinusoid impression of the keel–body transition, linear-lancolate valves, both apical and transapical torsion and elliptical to rectangular perforations within striae (Mejdandžić et al. 2017).

The last described species here, *Entomoneis umbratica*, has a very distinctive bilobate keel shape, an arcuate impression of the keel–body transition (as *E. adriatica*; Fig. 6, E and F) and intermediate fibulae forming an irregular line, as *E. japonica* and *E. pseudoduplex* (Osada and Kobayasi 1985, 1990c). Aside from the presence of intermediate fibulae, *E. umbratica* and *E. infula* differ in the shape of valve apices (scalpeliform in *E. infula*, but acute in *E. umbratica*) and number of poroids in abvalvar and adalvar valvocopulae striae (25–27 poroids in abvalvar, 6–9 poroids in advalvar striae for *E. infula* and 10–21 poroids in abvalvar, 7–14 poroids in advalvar striae for *E. umbratica*).

Comparisons to species outside the Adriatic Sea. Relative to other species included on the phylogeny, the Adriatic *Entomoneis* differ substantially in size (Adriatic species are generally much smaller, e.g., *E. adriatica* [maximal size 40.4 μm] and *E. vilicicii* [maximal size 38.4 μm]), the frustules are more lightly silicified, and have differently shaped transition between the elevated keel and valve body (e.g., undulate in *E. ornata*, sinusoid in *E. paludosa* and curved but not sinusoid in *E. pseudoduplex;* Patrick and Reimer 1975, Osada and Kobayasi 1990c).

Additionally, *Entomoneis paludosa* and *E. ornata* have monoseriate striae, while *Entomoneis pseudoduplex* has fine elongated perforations within hymenated stria, but those perforations are longer and the striae are wider than in the Adriatic species. Further differences reside in the cingulum morphology. *E. pseudoduplex* has poroids in areolae through the whole surface of the girdle bands, not just along the areolae edges. The cingulum of *E. paludosa* and *E. ornata* is similar to the Adriatic species, except that some Adriatic species have the peculiar silica thickenings or uniquely shaped valvocopulae (Patrick and Reimer 1975, Osada and Kobayasi 1990c). Comparisons to other *Entomoneis* species not included in our phylogeny is somewhat difficult, as many previous descriptions lack sufficient details, however, relative to *E. japonica*, *E. paludosa*, *E. punctulata*, *E. aequabilis,* and *E. vertebralis*, all Adriatic species are much smaller in size with less silicified cells therefore they can be easily identified (Osada and Kobayasi 1985, 1990c, 1991, Clavero et al. 1999).

Phylogeny and ecology of Adriatic Entomoneis. The data set compiled here with 39 strains with 2–3 genes, is the largest effort to date to reconstruct the phylogeny of *Entomoneis*. Our analyses of the concatenated SSU+*rbc*L+*psb*C data set, confirmed the monophyly of *Entomoneis* found previously (Ruck and Theriot 2011, Ruck et al. 2016, Mejdandžić et al. 2017) and reconstructed *Entomoneis* into two large clades, one dominated by strains from the Adriatic, and another by a number of unidentified strains together with *E. paludosa*, *E. ornata,* and *E. pulchra*. Apart from *rbc*L, single-gene trees did not recover a monophyletic *Entomoneis*, which was surprising given the unique set of morphological features typical for this genus and absent from the remaining Surirellales. The relationships relevant for this nonmonophyly, however, were not strongly supported, and topological hypothesis with the Shimodaira-Hasegawa and Approximately Unbiased tests did not reject the monophyly of *Entomoneis*.

Most differences between the single-gene phylogenies resided in how consistently recovered and well-supported clusters of *Entomoneis* were related to each other, but there were cases where clusters recovered with chloroplast data were not reconstructed with SSU (Fig. S1). For example, although a clade that included *E. pusilla* and *E. gracilis* was recovered with both chloroplast genes, these taxa were distantly related in the SSU tree (Figs. S1–S3). Discrepancies between gene trees, like the case of monophyly of *Entomoneis* discussed above, were not strongly supported (Figs. S1–S3). The mean of bootstrap values from the SSU phylogeny (~69.5%) was 10 (compared to *psb*C) and 12 (compared to *rbc*L) units lower than the mean bootstrap support value of the chloroplast data, suggesting that the relationships inferred with SSU data were more uncertain. Corroborating this, the internal branches were on average shorter in the SSU tree than in the chloroplast trees. It is possible, therefore, that the SSU alignment was not as informative as the chloroplast genes.

In both the BI and ML analyses of the concatenated data set, all Adriatic species fell in a single clade, and many of them were sister to each other (Fig. 1, A and B). However, some species shared a recent common ancestry with geographically distant isolates from the Arabian Sea, California, Guam, Tasmania, and South Africa (Fig 1, A and B; Table 1). Although detailed morphological and morphometric data needed to classify these geographically distant strains are lacking, at the molecular level some of them are evidently very similar (short branch lengths) to newly described species from the Adriatic (Fig. 1, A and B). These results could simply reflect the poor sampling of global diversity of *Entomoneis*, or alternatively, suggest that some Adriatic species might have wider geographic distributions. Another important question concerning these geographically distant strains within the clade populated with Adriatic species pertains to whether or not those strains were isolated from offshore plankton, like the ones in the Adriatic, or originated from shallower, coastal or estuarine habitats. At least some of the disjunct strains, i.e., the ones isolated from the Kariega River, South Africa and the shallow reef Gab Gab, Guam (Table 1), do not originate from typical planktonic habitats. However, given that they are represented by a single strain each, we cannot tell whether their ecological preferences are limited to littoral habitats or are broader and include the plankton. It is similarly possible that the Adriatic taxa are not exclusively planktonic. Better account of the diversity of *Entomoneis* in the benthos of the Adriatic Sea might reveal whether or not the pelagic species studied here are restricted to the plankton, or have broader ecological, and geographic, distributions.

The discovery of morphologically diagnosable species of *Entomoneis*, from a habitat not usually associated with a high diversity of raphid diatoms, highlights how underappreciated the diversity of planktonic raphid diatoms might be. In some ways, these *Entomoneis* species might resemble a case of “cryptic” diversity, as has been found, for example in *Skeletonema*, *Pseudo–nitzschia*, *Cyclotella*, *Chaetoceros* (Beszteri et al. 2005, Amato et al. 2007, Kooistra et al. 2008, Lundholm et al. 2012, Li et al. 2017). However, our interpretation of these findings is different because once tools that provide appropriate resolution were applied to circumscribe the morphology of the cultivated strains; it became apparent that there was nothing hidden or cryptic about the Adriatic *Entomoneis*. Even the conserved molecular markers used here were in most cases able to recover monophyly of the morphologically cohesive groups. Note that the development of these molecular markers was originally intended for questions at the all-diatom or at most order-level phylogenies (Medlin et al. 1993, Alverson et al. 2007, Theriot et al. 2010) and although these genes have been used in barcoding studies (e.g., Hamsher et al. 2011), they are not necessarily expected to work well at species or population level. The diversity of Adriatic *Entomoneis* is therefore cryptic only in the sense that no one had taken a closer look at these species in this particular habitat.

Perhaps a more intriguing question concerning the description of closely related species like these pertains to the amount of variation in morphology or sequence that corresponds to within-species phenotypic and genetic diversity. In general, microbial species, especially in the marine plankton, have large population sizes, broad geographic distributions, and plentiful opportunity for (passive) dispersal (Whittaker and Rynearson 2017). It is therefore, expected that such species might also exhibit comparatively high genetic and even phenotypic diversity as a result any number of reasons including local adaptation or stochastic population variation. Morphological differences related to phenology (e.g., Davey 1987) and phenotypic plasticity (e.g., Hasle et al. 1971, Schultz 1971, Leterme et al. 2013) are other sources of variation that frequently go unnoticed because sampling, especially of pelagic diatoms, occurs infrequently and experiments to assess potential phenotypic plasticity are commonly not done prior to species descriptions. With these and other considerations in mind, it becomes increasingly difficult to determine criteria for species delimitation (i.e., how much variation within and between populations are we willing to allow before describing a new diatom species). Here, we have taken a rather fine-grained approach in species delimitation that is corroborated by detailed morphological observations and phylogenetic analyses. However, we cannot rule out that some of the morphological differences we have attributed to different species are, in fact, part of the variation found within species. Further studies will undoubtedly offer a more complete view of the geographic distributions, morphological variation, ecological preferences, and plasticity of planktonic *Entomoneis* in the Adriatic Sea and beyond.

This material is based in part upon the work supported by Croatian Science Foundation under the project BIOTA (Bio–tracing Adriatic Water Masses), UIP-2013-11-6433 and by the National Science Foundation (NSF) under grant no. DEB-1353131. S.O. was funded by EU project STIM - REI, No.KK.01.1.1.01.0003. The authors declare there are no conflicts of interests. The authors are grateful to Petar Kružić and Nature park “Telašćica” for sampling campaigns in the central Adriatic Sea, and to crew of the RV “Naše more” during sampling cruises in the South Adriatic Sea. M.M. is grateful to Tanja Vojvoda Zeljko for help with laboratory work. Computations were performed at the Arkansas High Performance Computing centre. We appreciate the suggestions of the editor and constructive comments provided by two reviewers which greatly improved our manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Consensus ML phylogram constructed from SSU rDNA alignment containing 44 partial SSU rDNA sequences. Bootstrap values are indicated above branches, values below 50 not shown.

Figure S2. Consensus ML phylogram constructed from *psb*C alignment containing 42 partial *psb*C sequences. Bootstrap values are indicated above branches, values below 50 not shown.

Figure S3. Consensus ML phylogram constructed from *rbc*L alignment containing 48 partial *rbc*L sequences. Bootstrap values are indicated above branches, values below 50 not shown.

Figure S4. (A) Recently divided cells of *Entomoneis pusilla* sp. nov. with one plate-like plastid. (B) Panduriform cell of *E. gracilis* sp. nov. with one plate-like plastid; (C) Slender cell of *E. gracilis* sp. nov. with distinct straight transition between the keel and valve body. (D) Panduriform cell of

E. vilicicii sp. nov. with one plate-like plastid and lipid globules and straight to slightly arcuate transition between the keel and valve body. (E) Two cells of *E. infula* sp. nov. torsioned to a folded, saddle-like shape with a single plate-like plastid. (F) Panduriform cell of *E. adriatica* sp. nov. with a single multi-lobed plastid. (G) Torsioned cell of *E. adriatica* sp. nov.. (H) Cell of *E. umbratica* sp. nov. twisted around the apical axis with a single multi-lobed plate plastid. Scale bars = 10 µm.

Table S1. Primers used to amplify SSU rDNA, *rbc*L and *psb*C fragments in this study. Primers in bold were used for nested PCR reaction.

Table S2. Morphological features of six new *Entomoneis* species in comparison to similar species: *E. japonica, E. paludosa, E. punctulata,* and *E. tenera.* nd – not defined; np – not present.

Supplementary Material

Table S1. Primers used to amplify *SSU* rDNA, *rbcL* and *psbC* fragments in this study.

Primers in bold were used for nested PCR reaction.

Primer name	**Primer sequence (5'–3')**	**Reference**
SSU1	AAC CTG GTT GAT CCT GCC AGT	Medlin et al. 1988
ITS1DR	CCT TGT TAC GAC TTC ACC TTC C	Edgar & Theriot 2004
SSU11+	TGA TCC TGC CAG TAG TCA TAC GCT	Alverson et al. 2007
SSU1672–	TAG GTG CGA CGG GCG GTG T	Ruck & Theriot 2011
*rbc*L66+	TTA AGG AGA AAT AAA TGT CTC AAT CTG	Alverson et al. 2007
dp7–	AAA SHD CCT TGT GTW AGT YTC	Daugbjerg & Andersen 1997
*psb*C+	ACA GGM TTY GCT TGG TGG AGT GG	Alverson et al. 2007
*psb*C–	CAC GAC CWG AAT GCC ACC AAT G	Alverson et al. 2007
***psb*C22+**	CGT GGT GAT ACA TAG TTA	Ruck & Theriot 2011
***psb*C1154–**	GCD CAY GCT GGY TTA ATG G	Ruck & Theriot 2011

Table S2. Morphological features of six new *Entomoneis* species in comparison to similar species: *E. japonica*, *E. paludosa*, *E. punctulata* and *E. tenera*. nd – not defined; np – not present

	Similar species				**New species**					
Feature	***E. japonica***	***E. paludosa***	***E. punctulata***	***E. tenera***	***E. gracilis***	***E. pusilla***	***E. vilicicii***	***E. infula***	***E. adriatica***	***E. umbratica***
Plastid in cell	nd	Two axial plastids	nd	One, multi–lobed plastid	One plate–like plastid	One plate–like plastid	One plate–like plastid	One plate–like plastid	One, multi–lobed plastid	One multi–lobed plastid
Frustule shape	Panduriform	Panduriform	Panduriform	Panduriform, twisted around apical axis	Panduriform, rarely twisted around apical axis	Panduriform	Panduriform, twisted around apical axis only at one wing for 90°.	Panduriform, twisted around transapical or apical axis	Panduriform, twisted around apical axis	Panduriform, twisted around apical axis
Shape of transition between the keel and valve body	Bi–sinuous	Sinusoid	Arcuate, a short row of puncta, restricted to the end corner of the keel	Straight to slightly arcuate	Straight	Straight	Straight to slightly arcuate	Arcuate to slightly sinusoid	Arcuate	Arcuate
Valve length (µm)	75–150	40–130	18–99	11–22	13.2–36.0	9.7–14.1	15.8–38.4	16.8–27.8	11.3–40.4	7.2–27.0

Valve width (μm)	20–40	20–50	10–19	3–7	2.2–10.0	2.3–3.8	3.6–9.1	4.9–7.2	4.9–8.0	5.2–8.6
Valve striation	Parallel, biseriatae, 11–12 stria in 10 μm	Parallel, uniseriatae, 21–26 stria in 10 μm	Parallel, 34–36 stria in 10 μm	Not visible in LM, in EM parallel, 30–50 stria in 10 μm on valve body, 18–42 in 10 μm on the keel	Not visible in LM, in EM parallel, 35–45 stria in 10 μm on valve body, 40–52 in 10 μm on the keel	Not visible in LM, in EM parallel, 40–55 stria in 10 μm on valve body, 57–60 in 10 μm on the keel	Not visible in LM, in EM parallel, 40–50 stria in 10 μm on valve body, 38–45 in 10 μm on the keel	Not visible in LM, in EM parallel, 35–50 stria in 10 μm on valve body, 40–55 in 10 μm on the keel	Not visible in LM, in EM parallel, 40–60 stria in 10 μm on valve body, 45–50 in 10 μm on the keel	Not visible in LM, in EM parallel, 40–55 stria in 10 μm on valve body, 40–55 in 10 μm on the keel
Valve apex	Acuminate	Acute	Acute	Scalpeliform	Broad scalpeliform	Scalpeliform	Acute	Scalpeliform	Scalpeliform	Acute
Valve shape	Linear–lanceolate	Broad linear	Broad linear	Broad lanceolate	Narrowly lanceolate	Lanceolate	Narrowly lanceolate	Linear–lanceolate	Linear–lanceolate	Linear–lanceolate
Keel shape	Strongly sigmoid	Sigmoid, slightly torsioned	Sigmoid	Sigmoid, often strongly torsioned	Sigmoid, rarely torsioned	Sigmoid, never torsioned	Sigmoid, often torsioned in wing area	Sigmoid, often torsioned	Sigmoid, often torsioned	Sigmoid, often torsioned
Raphe fibulae	+	+	+	+, 29–40 in 10 μm	+, 42–55 in 10 μm	+, 50–60 in 10 μm	+ 40–50 in 10 μm	+ 38–40 in 10 μm	+ 40–50 in 10 μm	+ 40–45 in 10 μm
Keel fibulae	At several levels	nd	np	np	np	np	np	Intermediate fibulae	np	Intermediate fibulae

								scattered over keelsurface, 4–6 in 1 µm		forming an irregular line, 4–6 in 1 µm
Basal fibulae	+	+	Several in apical corner	+, 5–6 in 1 µm	+, 5–6 in 1 µm	+, 4–5 in 1 µm	+, 4–6 in 1 µm	+, 4–5 in 1 µm	+, 4–5 in 1 µm	+, 4–5 in 1 µm
Striae perforation	Two rows of poroid areolae occluded by perforated hymen (hymenate pore occlusion)	Two rows of elliptical poroid areolae, closed externally with hymen, 18–25 in 10 µm within valve body stria, 22–40 areolae within keel stria in 10 µm	Hymen perforated with parallel marginal linear perforations	Hymen with rectangular perforations arranged in two parallel lines along the stria edges; 26–37 in 1 µm near keel margin and 20–39 in 1 µm near the valve margin	Hymen with elongated perforations arranged in two parallel lines along the stria edges; 32–41 in 1 µm near keel margin and 36–41 in 1 µm near the valve margin	Hymen with narrow dash-like perforations arranged in two parallel lines along the stria edges; 64–75 in 1 µm near keel margin and 49–50 in 1 µm near the valve margin	Hymen with round to elliptical perforations arranged in two parallel lines along the stria edges; 32–38 in 1 µm near keel margin and 24–34 in 1 µm near the valve margin	Hymen with round to elliptical perforations arranged in two parallel lines along the stria edges; 31–35 in 1 µm near keel margin and 26–32 in 1 µm near the valve margin	Hymen with round to elliptical perforations arranged in two parallel lines along the stria edges	Hymen with round to elliptical perforations arranged in two parallel lines along the stria edges; 24–29 in 1 µm near keel margin and 24–32 in 1 µm near the valve margin

No. of cingulum bands / girdle appearance	5 open bands / nd	5–6 open bands / crossed	5–6 open bands / crossed	4–5 open bands / crossed	3–4 open bands / straight	4 open bands / straight	4 open bands / straight but few bands in the middle crossed	4 open bands / crossed	4 open bands / crossed	4 open bands / crossed
Valvocopulae ultrastructure	nd	nd	nd	nd	Striae (56–60 in 10 µm),; 31–42 poroids in abvalvar, 6–8 poroids in advalvar striae	Two rows of distinct teardrop shaped areolae (40–45 in 10 µm) with more elongated drop apex and larger radius in abvalvar than in advalvar ones; 14–29 poroids in advalvar, 24–32 poroids	Valvocopulae clepsydriform. Striae (55–60 in 10 µm),; 19–44 poroids in abvalvar, 10–17 poroids in advalvar striae	Striae (50–60 in 10 µm), 25–27 poroids in abvalvar, 6–9 poroids in advalvar striae	Striae (50–65 in 10 µm),	Striae (50–65 in 10 µm), 10–21 poroids in abvalvar, 7–14 poroids in advalvar striae

						in abvalvar areolae				
Copula ultrastructure	Two rows of areolae, abvalvar elongated, advalvar short	Two rows of poroids: abvalvar elongated advalvar circular	Two rows of areolae: Advalvar shorter than abvalvar in bands near the valve, almost equal in the abvalvar bands	Two rows of elongated areolae: 56–60 in 10 μm	Striae: 60–70 in 10 μm	Two rows of distinct teardrop shaped areolae (40–45 in 10 μm)	Striae: 50–60 in 10 μm	Striae: 50–60 in 10 μm	Striae: 50–65 in 10 μm	Striae: 55–70 in 10 μm
Reference	**Osada & Kobayasi 1985**	**Osada & Kobayasi 1990c**	**Osada & Kobayasi 1990c**	**Mejdandžić et al. 2017**	**This study**	**This study**	**This study**	**This study**	**This study**	**This study**

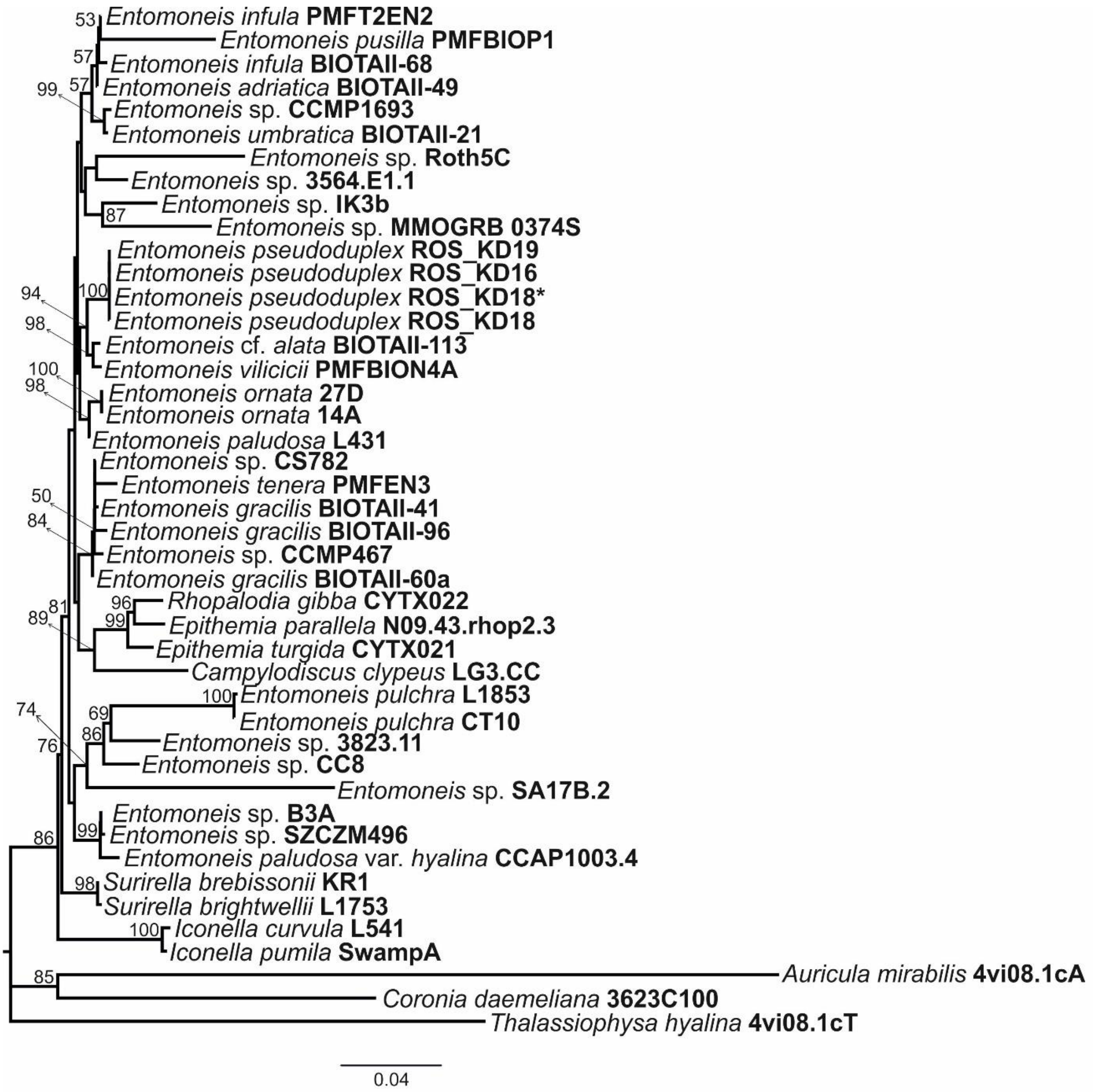

Figure S1. Consensus ML phylogram constructed from SSU rDNA alignment containing 44 partial SSU rDNA sequences. Bootstrap values are indicated above branches or with arrows, values below 50 not shown.

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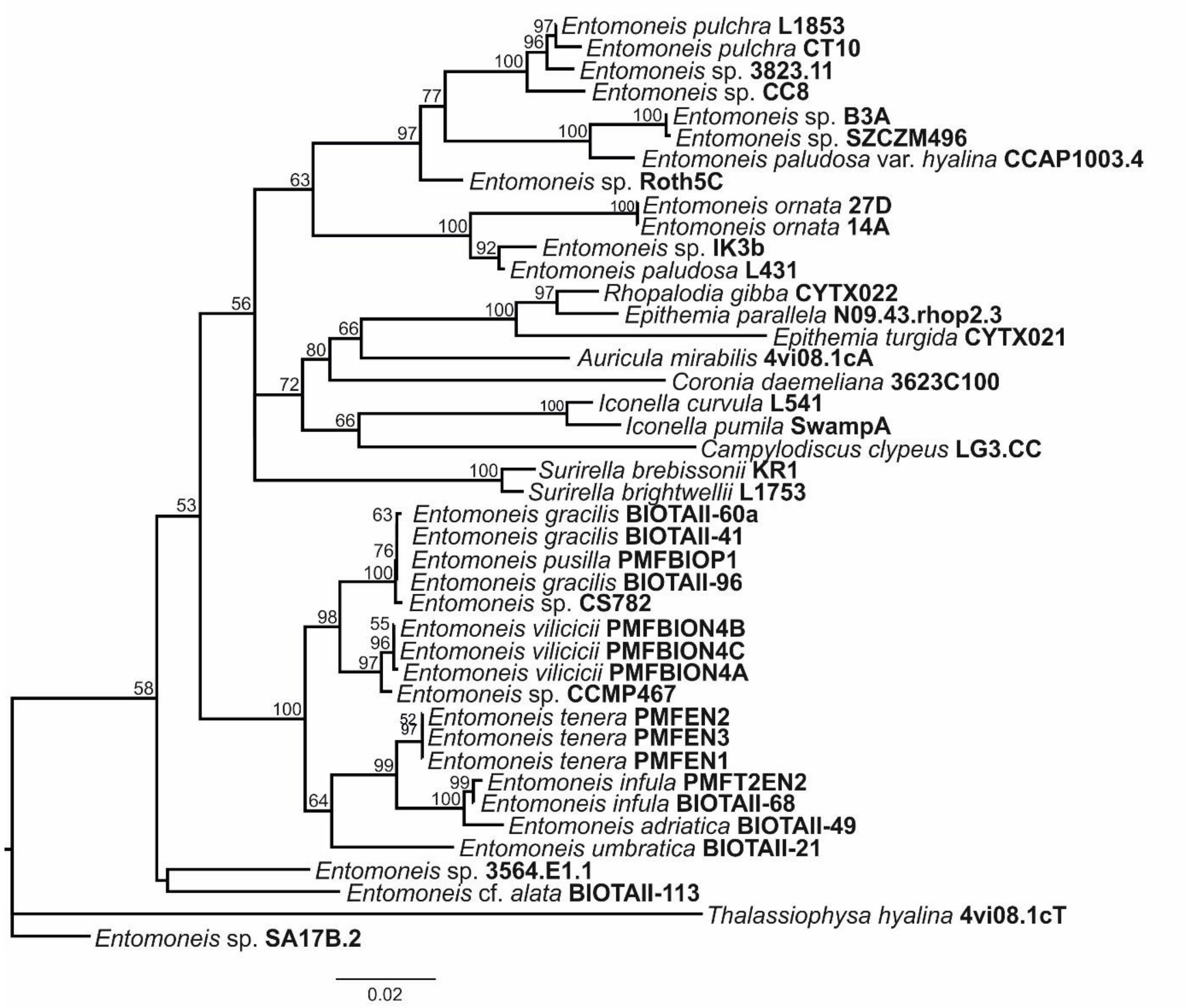


Figure S2. Consensus ML phylogram constructed from psbC alignment containing 42 partial psbC sequences. Bootstrap values are indicated above branches, values below 50 not shown.

Figure S2. Consensus ML phylogram constructed from *psb*C alignment containing 42 partial *psb*C sequences. Bootstrap values are indicated above branches, values below 50 not shown.

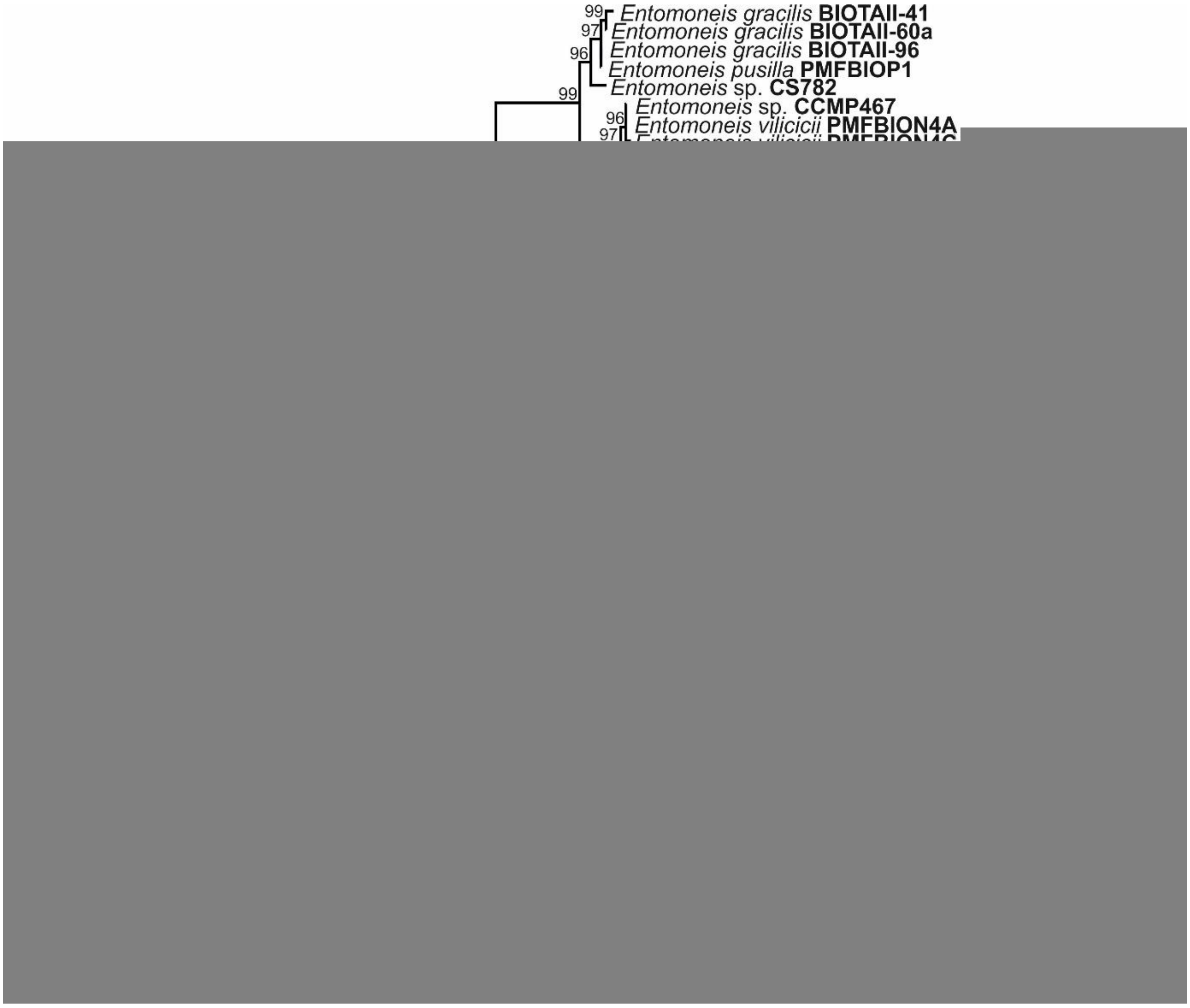
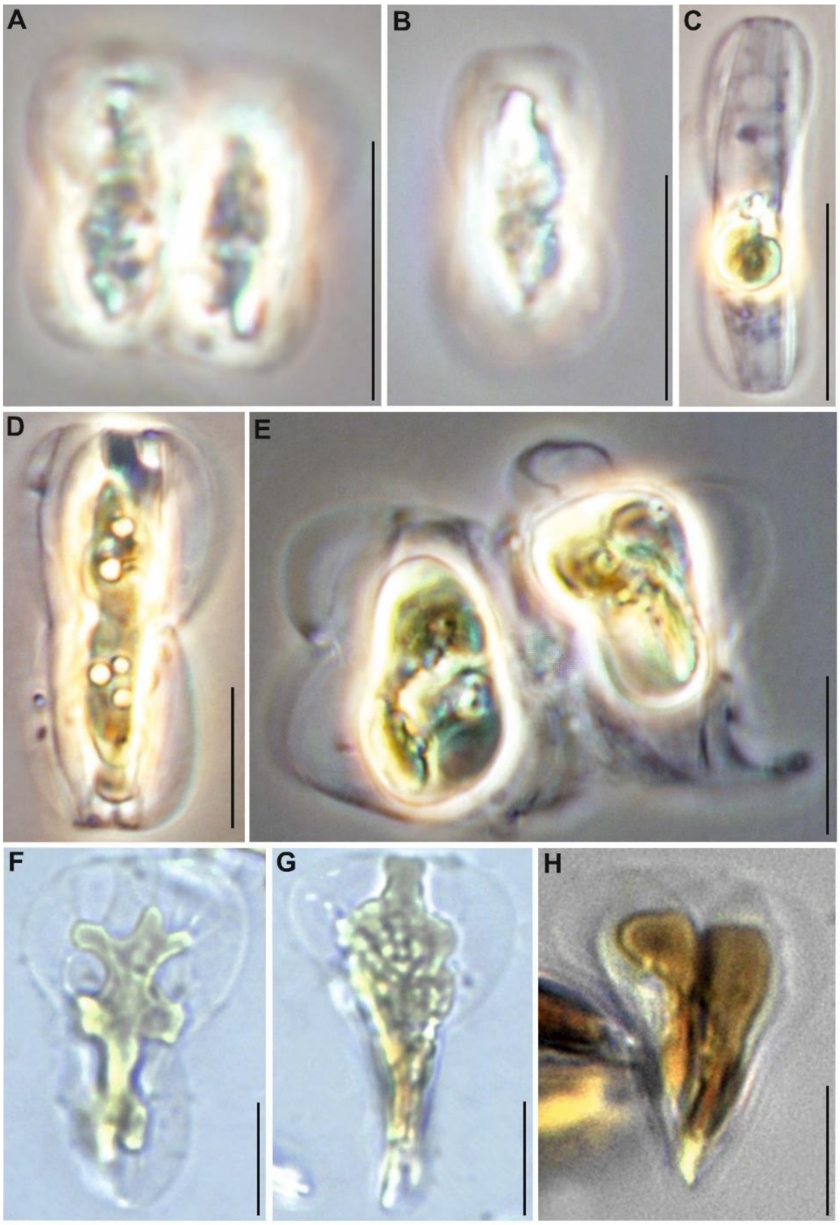


Figure S3. Consensus ML phylogram constructed from *rbc*L alignment containing 48 partial *rbc*L sequences. Bootstrap values are indicated above branches, values below 50 not shown.



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Publication III

Diatom Research, 2017
Vol. 32, No. 1, 127–131, http://dx.doi.org/10.1080/0269249X.2017.1284158

NOTE

Multigene phylogeny and morphology of newly isolated strain of *Pseudo-nitzschia mannii* Amato & Montresor (Adriatic Sea)

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An increasing number of cryptic and pseudo-cryptic species have been found within many newly described diatom species. To resolve the phylogenetic relationships of the genus *Pseudo-nitzschia*, molecular markers are being widely used in combination (or separately) with different morphological characters. Sequence analysis of ribosomal DNA markers (18S, ITS and 28S) and morphological analyses of *Pseudo-nitzschia mannii* strain (CIM_D-4), isolated from the Telašćica Bay (Adriatic Sea), differentiate it from all other currently reported strains of this species.

Keywords: *molecular markers, morphology, phylogeny, phytoplankton,* Pseudo-nitzschia

Introduction

Genetically distinct groups can exist within phytoplankton species described only by morphological characters (Amato et al. 2007). A combination of molecular markers (i.e. nuclear ribosomal genes (rDNA), internal transcribed spacer (ITS) regions, and mitochondrial and cytochrome oxidase genes) have been widely used to resolve the phylogenetic structure of *Pseudo-nitzschia* H. Peragallo in H. & M. Peragallo (Lundholm et al. 2002, Lim et al. 2014, Tan et al. 2015, Lim et al. 2016).

Until recently, only partial sequences of 28S rDNA (domains D1–D3) and the fast evolving, highly variable ITS1-5,8S-ITS2 (ITS) regions were used in phylogenetic analyses of *Pseudo-nitzschia* (Lim et al. 2016). In diatoms, 18S rDNA regions have conservative rates of evolution and are considered inadequate for analysing phylogenetic relationships except at higher taxonomic levels (Medlin et al. 1993, Kooistra & Medlin 1996, Medlin et al. 1996, Sorhannus 1997, 2007, Zhang et al. 2007, Alverson 2008, Medlin et al. 2008, Theriot et al. 2009, Moniz & Kaczmarska 2010, Lundholm et al. 2012). Yet, as Lim et al. (2016) have demonstrated, by incorporating all three rDNA markers in the analyses, 18S rDNA can provide important additional information.

Here we document the morphology of *Pseudo-nitzschia mannii* Amato & Montresor, strain CIM_D-4, isolated from Telašćica Bay (Adriatic Sea) alongside a multigene phylogeny inferred from the obtained 18S, ITS and 28S rDNA sequences.

Material and methods

Sampling

Net phytoplankton samples (20 µm-pore-size mesh) were collected from inner Station T4 in Telašćica Bay (Adriatic Sea, Fig. S1, Supplement material) in August 2012. A strain generated from single cells (or clonal chain of cells) of *P. mannii* was isolated and maintained as a monoclonal culture (strain CIM_D-4) in Guillard's f/2 marine water enrichment solution (Sigma-Aldrich) with a 12:12 h light dark cycle and constant temperature (Guillard 1983). Cultured cells were analysed morphologically with LM and transmission electron microscopy (TEM).

For TEM and preparation of permanent slides, *Pseudo-nitzschia* frustules were first acid-cleaned (combination of HNO_3 and H_2SO_4) and rinsed with distilled water. Cleaned frustules in distilled water were mounted on formvar/carbon coated copper grids and micrographs were

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Associate Editor: Eileen Cox

(Received 7 June 2016; accepted 21 December 2016)

taken with a FEI Morgagni 268D transmission electron microscope. Permanent slides for morphometry with light microscopy were made from cleaned samples mounted in Zrax. Morphometric values for *P. mannii* (strain CIM_D-4) were compared with other studies (Table 1).

Genetic characterization and phylogenetic analysis

DNA was isolated from the monoclonal culture of *P. mannii* (strain CIM_D-4) with a Qiagen plant tissue kit (Qiagen GmgH, Hilden, Germany) according to the manufacturer's instructions.

The hypervariable region of the small subunit (SSU) 18S ribosomal DNA (rDNA) gene was amplified using the primer set D512for 18S and D978rev 18S (Table S2) according to Zimmermann et al. (2011). Additionally, we used primer set ITSL and Diat-ITS-NL38-R (Table 2) to amplify the ITS region (ITS1-5,8S-ITS2) as described in Lundholm et al. (2003) and primer set D1R and D3Ca (Table S2) to amplify partial large subunit (LSU) 28S rDNA as described in Amato et al. (2007).

All nucleotide sequences were obtained commercially from Macrogen (Amsterdam, The Netherlands), using the sequencing Big Dye TM Terminator Kit and ABI 3730XL (Applied Biosystems). Retrieved 18S rDNA sequences from two runs in each direction were compared in order to exclude sequencing mistakes by majority rule (3:1), resulting a 760 base pair (bp) long 18S rDNA sequence (SSU sequence; V4 region). For ITS a 838 bp long sequence (ITS1-5,8S-ITS2 region) was obtained, and for 28S rDNA a 786 bp long sequence (LSU sequence; D1–D3 domains).

All three newly obtained sequences of *P. mannii* Telašćica strain CIM_D-4 were deposited in GenBank under the following accession numbers: KX215915 for 18S rDNA; KX215916 for ITS, and KX215917 for 28S rDNA.

Three datasets including *P. mannii* Telašćica strain CIM_D-4 were analysed: nuclear 18S rDNA, ITS and 28S rDNA. The 18S rDNA sequence alignment included 22 sequences – 20 of *Pseudo-nitzschia* taxa, one sequence each for *Nitzschia* Hassall and *Cylindrotheca* Rabenhorst. The ITS sequence alignment included 23 sequences – 21 for *Pseudo-nitzschia* and 1 sequence each for *Nitzschia* and *Cylindrotheca*. The 28S rDNA alignment included 19 sequences – 17 *Pseudo-nitzschia* species/strains and 2 for *Nitzschia*. All selected sequences were obtained from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) using a basic alignment search tool (tblastn) and are listed in the Supplementary Tables S1, S2 and S3. Each multiple sequence alignment was performed using Clustal X version (v) 2.0 (Larkin et al. 2007) and corrected and manually refined using BioEdit v 7.0.5.3 (Hall 1999).

Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were performed using MEGA 6 software (Tamura et al. 2013). MP analyses were carried out using heuristic searches with the random addition of sequences (1000 replicates), and branch-swapping with tree-bisection-reconnection (TBR) (Nei & Kumar 2000). ML analyses were performed using heuristic searches with ten random addition replicates and the TBR branch-swapping algorithm. The best-fitting evolutionary models were identified from the lowest BIC scores (Bayesian Information Criterion) that included AICc values (Akaike Information Criterion) (Tamura et al. 2013). For all three datasets the best-fitting evolutionary model was K2 + G (Kimura 2-parameter + discrete Gamma distribution). The reliability of the phylogenetic relationships

Table 1. Comparison of morphometric data on *P. mannii* strains.

Length (µm)		Width (µm)		Fibulae/10 µm		Striae/10 µm		Poroids/µm		Divided sector		
Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Reference
57	89	1.3	1.8	18	26	37	44	4	6	2	5	This work
77	98	1.3	1.7	19	24	34	41	4	6	2	4	Ljubešić et al. (2011)
115	117	3.1	3.3	17	20	27	30	4	5	–	–	Quijano-Scheggia et al. (2010)
53	93	1.6	2.1	18	23	32	39	4	6	2	7	Moschandreou et al. (2010)
30	130	1.7	2.6	17	25	30	40	4	6	2	7	Amato and Montresor (2008)

Table 2. List of primers used in this study.

Region	Primer ID	Primers	Reference
18S	D512for 18S	5-ATTCCAGCTCCAATAGCG-3	Zimmermann et al. (2011)
	D978rev 18S	5-GACTACGATGGTATCTAATC-3	Zimmermann et al. (2011)
ITS1-5,8S-ITS2	ITSL	5-TCGTAACAAGGTTTCCGTAGGTG-3	Lundholm et al. (2003)
	Diat-ITS-NL38-R	5-CGCTTAATTATATGCTTA-3	Lundholm et al. (2003)
28S(D1-D2)	D1R	5-ACCCGCTGAATTTAAGCATA-3	Amato et al. (2007)
	D3Ca	5-ACGAACGATTTGCACGTCAG-3	Amato et al. (2007)

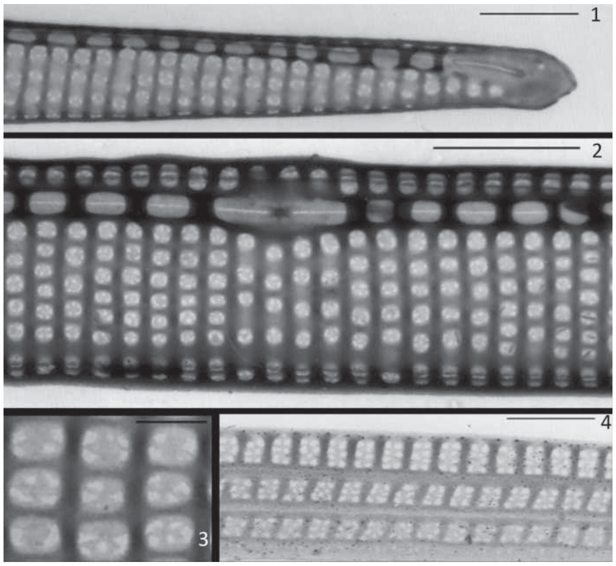
was evaluated using a non-parametric bootstrap analysis with 1000 replicates. Bootstrap values (BP) above 75 were considered well supported.

Bayesian inference (BI) analyses were also performed on all three datasets using MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003), each using default priors and the general time reversible (GTR) model with a gamma distribution (G) and a proportion of invariable sites (I) (GTR + G + I) model. Posterior probabilities were assessed in 2 runs, using 4 MCMC chains with trees (5 million generations, sampling every 1000th generation, burn-in period 500,000). Stationarity was confirmed using Tracer ver. 1.5 (Drummond & Rambaut 2007). Finally, consensus phylogenetic trees were generated using FigTree v.1.4.2. (available at tree.bio.ed.ac.uk/software/figtree/), including Bayesian posterior probability (BPP), MP and ML BP at branch nodes.

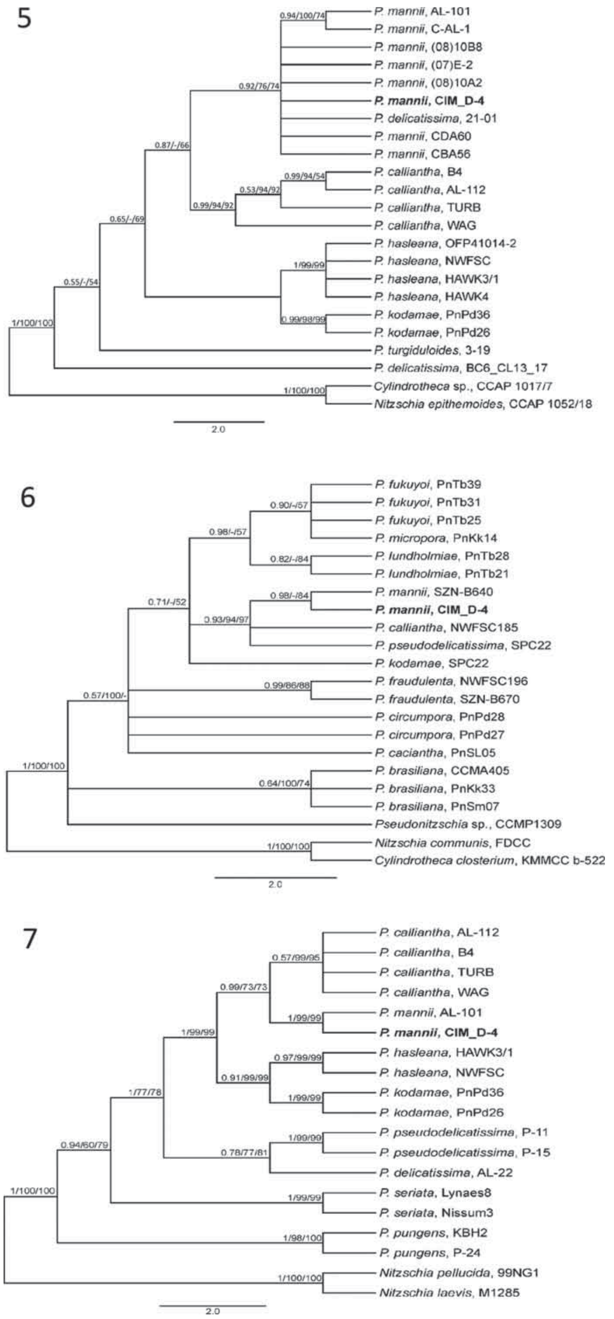
Results and discussion

Cells isolated from Telašćica Bay as a monoclonal culture (strain CIM_D-4) were confirmed as *P. mannii*. by morphological and molecular analysis As found by Ljubešić et al. (2011), morphological measurements showed that the cells were slightly narrower (1.3–1.8 µm) than in the type material (1.7–2.6 µm), but still match the original description (Amato & Montresor 2008) (Table 1). Since all other morphological characteristics and measurements corresponded to the original description, the culture was identified as *P. mannii* (Figs 1–4).

Phylogenetic analyses performed on the obtained 18S (KX215915, 760 bp, V4 region), ITS (KX215916, 838 bp, ITS1-5,8S-ITS2 region) and 28S (KX215917, 786 bp, D1–D3 region) sequences confirmed the position of CIM_D-4 strain within the *P. mannii* clade (BPP: 0.98, 0.92 and



Figs 1–4. *P. mannii*, TEM. Tip of valve (Fig. 1). Scale bar: 1 µm. Central part of the valve (Fig. 2). Scale bar: 1 µm. Perforation pattern of the areolae (Fig. 3). Scale bar: 0.5 µm. Girdle bands (Fig. 4). Scale bar: 0.25 µm.



Figs 5–7. Phylogenetic position of CIM_D-4 (*P. mannii* Telašćica strain) based on ITS rDNA gene sequence data (23 taxa) (Fig. 5), 18S rDNA gene sequence data (22 taxa) (Fig. 6), and 28S rDNA gene sequence data (19 taxa) (Fig. 7). The trees were rooted with two raphid taxa. BPP and BP values greater than 50 are shown on the nodes that were recovered with BI analysis (GTR + G + I model, 5M generations with burn-in 500,000, MLE –ln L = -9134.029), Maximum likelihood (ML) analysis (K2 + G model, 1000 replicates of bootstrap) and MP analysis (1000 replicates of bootstrap). Taxa in bold designate sequence obtained in this study.

1 respectively) (Figs 5–7). Our Telašćica strain CIM_D-4 18S sequence matched the KJ608080 sequence (strain SZN-B640), which is unpublished but annotated as *P. mannii* in NCBI GenBank database. This confirms that 18S rDNA (Fig. 6) is also useful for exploring intrageneric relationships, as recently shown by Lim et al. (2016). As shown on the 28S phylogenetic tree (Fig. 7), the *P. mannii* Telašćica strain CIM_D-4 28S rDNA sequence matched the earlier published sequence DQ813814 (strain AL-101) (Amato & Montresor 2008). Finally, ITS rDNA sequence (Fig. 5) grouped with seven other strains that together form the *P. mannii* clade, which also included one *Pseudo-nitzschia delicatissima* (Cleve) Heiden in Heiden & Kolbe sequence (strain 21-01, accession number AY519274). The presence of a *P. delicatissima* sequence within the *P. mannii* clade emphasizes the importance of combining analyses (e.g. morphological and molecular) and exploring phylogenetic relationships in order to obtain correct identifications of *Pseudo-nitzschia* species.

Pseudo-nitzschia caliantha Lundholm, Moestrup & Hasle was the most similar species to *P. mannii*, distinguished by a well-supported branch with BPP of 0.87 on ITS (Fig. 5) and 0.99 on the 28S tree (Fig. 7). Furthermore, phylogenetic analyses revealed that the *P. mannii* and *P. caliantha* clades cluster separately, away from other *Pseudo-nitzschia* species (*P. kodamae*, *P. hasleana*, *P. seriata* and *P. delicatissima*) (Figs 5–7).

In conclusion, morphological and phylogenetic analyses of *P. mannii* Telašćica strain CIM_D-4 discriminate a separate, indigenous population of *P. mannii* in the middle of the Adriatic Sea in Telašćica Bay.

Acknowledgements

The authors would like to express their thanks to Katrina O'Loughlin for critical reading of the manuscript and language corrections. Special gratitude goes to two anonymous reviewers whose comments and suggestions helped improve this manuscript. The authors would like to thank the Nature park 'Telašćica' office for their financial support of the research, and assistance with sampling. The study was supported in part by the Croatian Ministry of Science, Education and Sports (project no. 119-1191189-1228) and by the Croatian Science Foundation (project no. UIP-11-2013-6433).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Hrvatska Zaklada za Znanost [grant number UIP-11-2013-6433]; Ministarstvo Znanosti, Obrazovanja i Sporta [grant number 119-1191189-1228].

Supplemental data

Supplemental data for this article can be accessed http://dx.doi.org/10.1080/0269249X.2017.1284158.

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Supporting Information

Multigene phylogeny and morphology of newly isolated strain of Pseudo-nitzschia mannii Amato & Montresor (Adriatic Sea)

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Supporting Information consists of 12 SI pages (S1 – S12), 5 SI Tables (Table S1 – S5) and one SI Figures (Figures S1).

Material and Methods

Morphological characterization

For transmission electron microscopy (TEM) and preparation of permanent slides, *Pseudo-nitzschia* frustules were first acid-cleaned (combination of HNO_3 and H_2SO_4) and rinsed with distilled water. Cleaned frustules in distilled water were mounted on the copper grid and micrographs were taken with a FEI Morgagni 268D transmission electron microscope. Permanent slides for morphometry on a light microscope were made from cleaned samples mounted in Zrax.

Genetic characterization

DNA was isolated from *P. mannii* monoclonal culture (strain CIM_D-4) with a Qiagen plant tissue kit (Qiagen GmgH, Hilden, Germany) according to the manufacturer's instructions.

The hypervariable region of the small subunit (SSU) 18S ribosomal DNA (rDNA) gene was amplified using the primer set D512for 18S and D978rev 18S (Table S2) according to Zimmermann et al. (2011). Additionally, we used primer set ITSL and Diat-ITS-NL38-R (Table S2) to amplify the internal transcribed spacer (ITS) region (ITS1 -5,8S-ITS2) as described in Lundholm et al. (2003) and primer set D1R and D3Ca (Table S2) to amplify partial large subunit (LSU) 28S rDNA as described in Amato et al. (2007).

All nucleotide sequences were commercially obtained by submission to Macrogen (Amsterdam, The Netherlands), using the sequencing Big Dye TM Terminator Kit and ABI 3730XL (Applied Biosystems). Retrieved 18S rDNA sequences from two runs for each direction were compared in order to exclude sequencing mistakes by majority rule (3:1) resulting in 760 base pair (bp) long 18S rDNA sequence (SSU sequence; V4 region). For ITS the result was 838 bp long sequence (ITS1–5,8S–ITS2 region) and for 28S rDNA 786 bp long sequence (LSU sequence; D1–D3 domains).

All three newly obtained sequences of *P. mannii* Telašćica strain CIM_D-4 were deposited in the GenBank under the following accession numbers: KX215915 for 18S rDNA; KX215916 for ITS, and KX215917 for 28S rDNA.

Phylogenetic analysis

Three datasets, including *P. mannii* Telašćica strain CIM_D-4, were analyzed: the nuclear 18S rDNA, ITS and 28S rDNA. 18S rDNA sequence alignment included a total of 22 18S DNA sequences - twenty of the genus *Pseudo-nitzschia*, one sequence per genus *Nitzschia* and *Cylindrotheca*. ITS sequence alignment included a total of 23 ITS DNA sequences - 21 of the genus *Pseudo-nitzschia* and one sequence per genus *Nitzschia* and *Cylindrotheca*. 28S rDNA alignment included a total of 19 sequences – 17 different *Pseudo-nitzschia* species/strains and two of the genus *Nitzschia*. All selected sequences were obtained from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) by using a basic alignment search tool (tblastn) and are listed in Supplementary Tables S3, S4 and S5. Each multiple sequence alignment was performed using Clustal X version (v) 2.0 (Larkin et al. 2007) and subsequently corrected and manually refined using BioEdit v 7.0.5.3 (Hall 1999).

Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were performed by MEGA 6 software (Tamura et al. 2013). MP analyses were carried out using heuristic searches with the random addition of sequences (1000 replicates), and branch-swapping with tree-bisection-reconnection (TBR) (Nei & Kumar 2000). ML analyses were performed using heuristic searches with 10 random addition replicates and the TBR branch-swapping algorithm. The best-fitting evolutionary models were identified according to lowest BIC scores (Bayesian Information Criterion) that included AICc value (Akaike Information Criterion) (Tamura et al. 2013). For all three alignments (18S DNA, ITS and 28S) the best-fitting evolutionary model was K2+G (Kimura 2-parameter + discreet Gamma distribution). The reliability of phylogenetic relationships were evaluated using a non-parametric bootstrap analysis with 1000 replicates. The bootstrap values exceeding 75 were considered well supported.

Additionally, Bayesian inference (BI) analyses were performed using MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) on all three datasets, each using default priors and the general time reversible (GTR) model with a gamma distribution (G) and a proportion of invariable sites (I) (GTR+G+I) model. Posterior probabilities were assessed in two runs, using four MCMC chains with trees (5 million generations, sampling every 1000th generation, burn-in period 500000). Stationarity was confirmed using Tracer ver. 1.5 (Drummond & Rambaut 2007). Finally, consensus phylogenetic trees were made using FigTree v.1.4.2. (available at tree.bio.ed.ac.uk/software/figtree/), comprising Bayesian posterior probability (BPP), MP and ML bootstrap values (BP) presented at branch nodes.

TABLES

Table S1. Comparison of *Pseudo-nitzschia mannii* strains morphometry through studies.

Lenght (μm)		Widht (μm)		Fibulae/ 10μm		Striae/ 10μm		Poroids/ μm		Divided sector		Authors
Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	
57	89	1.3	1.8	18	26	37	44	4	6	2	5	This work
77	98	1.3	1.7	19	24	34	41	4	6	2	4	Ljubešić et al. 2011
115	117	3.1	3.3	17	20	27	30	4	5	-	-	Quijano-Scheggia et al. 2010
53	93	1.6	2.1	18	23	32	39	4	6	2	7	Moschandreou & Nikolaidis 2010
30	130	1.7	2.6	17	25	30	40	4	6	2	7	Amato & Montresor, 2008

Table S2. List of primers used in this study.

Region	Primer ID	Primers	Reference
18S	D512for 18S	5-ATTCCAGCTCCAATAGCG-3	Zimmermann et al. (2011)
	D978rev 18S	5-GACTACGATGGTATCTAATC-3	Zimmermann et al. (2011)
ITS1-5,8S-ITS2	ITSL	5-TCGTAACAAGGTTTCCGTAGGTG-3	Lundholm et al. (2003)
	Diat-ITS-NL38-R	5-CGCTTAATTATATGCTTA-3	Lundholm et al. (2003)
28S(D1-D2)	D1R	5-ACCCGCTGAATTTAAGCATA-3	Amato et al. (2007)
	D3Ca	5-ACGAACGATTTGCACGTCAG-3	Amato et al. (2007)

Table S3. Origins of the *Pseudo-nitzschia*, *Nitzschia* and *Cylindrotheca* 18S strains and DNA seqence GenBank accession number. Taxa in bold designate sequence obtained in this study.

Species	Strain	GenBank accession num.	Reference
Pseudo-nitzschia brasiliana	PnKk33	KP708991	Lim et al. 2016
Pseudo-nitzschia brasiliana	PnSm07	KP708990	Lim et al. 2016
Pseudo-nitzschia brasiliana	CCMA405	KM386874	Wang et al. Direct Submission
Pseudo-nitzschia caciantha	PnSL05	KP708992	Lim et al. 2016
Pseudo-nitzschia calliantha	NWFSC185	JN091716	Boardman et al. Direct Submission
Pseudo-nitzschia circumpora	PnPd28	KP708994	Lim et al. 2016
Pseudo-nitzschia circumpora	PnPd27	KP708993	Lim et al. 2016
Pseudo-nitzschia fraudulenta	SZN-B670	KJ608077	Ruggiero & Italiano, Direct Submission
Pseudo-nitzschia fraudulenta	NWFSC196	JN091721	Boardman et al. Direct Submission
Pseudo-nitzschia fukuyoi	PnTb39	KP708999	Lim et al. 2016
Pseudo-nitzschia fukuyoi	PnTb31	KP708998	Lim et al. 2016
Pseudo-nitzschia fukuyoi	PnTb25	KP708997	Lim et al. 2016
Pseudo-nitzschia kodamae	PnPd31	KP709000	Lim et al. 2016
Pseudo-nitzschia lundholmiae	PnTb28	KP709002	Lim et al. 2016
Pseudo-nitzschia lundholmiae	PnTb21	KP709001	Lim et al. 2016
Pseudo-nitzschia mannii	**CIM_D-4**	**KX215915**	**This study**
Pseudo-nitzschia mannii	SZN-B640	KJ608080	Ruggiero & Italiano Direct Submission
Pseudo-nitzschia micropora	PnKk14	KP709003	Lim et al. 2016
Pseudo-nitzschia pseudodelicatissima	isolate SPC22	GU373965	Fitzpatrick et al. 2010
Pseudo-nitzschia sp.	CCMP1309	GU373970	Fitzpatrick et al. 2010
Cylindrotheca closterium	KMMCC:B-552	GQ468545	Youn & Hu, Direct Submission
Nitzschia communis	FDCC L408	AJ867278	Rimet et al. Direct Submission

Table S4. Origins of the *Pseudo-nitzschia*, *Nitzschia* and *Cylindrotheca* ITS strains and DNA seqence GenBank accession number. Taxa in bold designate sequence obtained in this study.

Species	Strain	GenBank accession num.	Reference
Pseudo-nitzschia calliantha	B4	DQ530621	Andree, Direct Submission
Pseudo-nitzschia calliantha	TURB	KC017464	Ajani et al. 2013
Pseudo-nitzschia calliantha	WAG	KC017463	Ajani et al. 2013
Pseudo-nitzschia calliantha	AL-112	DQ813841	Amato et al. 2007
Pseudo-nitzschia delicatissima	BC6_CL13_17	KM245506	Noyer et al. 2015
Pseudo-nitzschia delicatissima	21-01	AY519274	Orsini et al. 2004
Pseudo-nitzschia hasleana	HAWK3/1	KC017450	Ajani et al. 2013
Pseudo-nitzschia hasleana	HAWK4	KC017468	Ajani et al. 2013
Pseudo-nitzschia hasleana	NWFSC 186	JN050282	Lundholm et al. 2012
Pseudo-nitzschia hasleana	OFP41014-2	JN050286	Lundholm et al. 2012
Pseudo-nitzschia kodamae	PnPd36	KF482053	Teng et al. 2014
Pseudo-nitzschia kodamae	PnPd26	KF482050	Teng et al. 2014
Pseudo-nitzschia mannii	**CIM_D-4**	**KX215916**	**This study**
Pseudo-nitzschia mannii	CBA60	HE650978	Penna et al. 2013
Pseudo-nitzschia mannii	CBA56	HE650977	Penna et al. 2013
Pseudo-nitzschia mannii	AL-101	DQ813839	Amato et al. 2007
Pseudo-nitzschia mannii	C-AL-1	DQ813842	Amato et al. 2007
Pseudo-nitzschia mannii	(08)10A2	JF714905	Moschandreou et al. Direct Submission
Pseudo-nitzschia mannii	(08)10B8	JF714904	Moschandreou et al. Direct Submission
Pseudo-nitzschia mannii	(07)E-2	JF714903	Moschandreou et al. Direct Submission
Pseudo-nitzschia turgiduloides	3-19	AY257839	Lundholm et al. 2003
Cylindrotheca sp.	CCAP 1017/7	FR865492	Heesch, Direct Submission
Nitzschia epithemoides	CCAP 1052/18	FR865501	Heesch, Direct Submission

Table S5. Origins of the *Pseudo-nitzschia* and *Nitzschia* 28S strains and DNA seqence GenBank accession number. Taxa in bold designate sequence obtained in this study.

Species	Strain	GenBank accession num.	Reference
Pseudo-nitzschia calliantha	TURB	KC017452	Ajani et al. 2013
Pseudo-nitzschia calliantha	WAG	KC017451	Ajani et al. 2013
Pseudo-nitzschia calliantha	B4	EF642976	Andree, Direct Submission
Pseudo-nitzschia calliantha	AL-112	DQ813815	Amato et al. 2007
Pseudo-nitzschia delicatissima	AL-22	DQ813810	Amato et al. 2007
Pseudo-nitzschia hasleana	HAWK3/1	KC017446	Ajani et al. 2013
Pseudo-nitzschia hasleana	NWFSC186	JN050298	Lundholm et al. 2012
Pseudo-nitzschia kodamae	PnPd36	KF482045	Teng et al. 2014
Pseudo-nitzschia kodamae	PnPd26	KF482042	Teng et al. 2014
Pseudo-nitzschia mannii	**CIM_D-4**	**KX215917**	**This study**
Pseudo-nitzschia mannii	AL-101	DQ813814	Amato et al. 2007
Pseudo-nitzschia pseudodelicatissima	P-11	AF417640	Lundholm et al. 2003
Pseudo-nitzschia pseudodelicatissima	P-15	DQ813808	Amato et al. 2007
Pseudo-nitzschia pungens	KBH2	AF417650	Lundholm et al. 2002
Pseudo-nitzschia pungens	P-24	AF417648	Lundholm et al. 2003
Pseudo-nitzschia seriata	Lynaes8	AF417653	Lundholm et al. 2002
Pseudo-nitzschia seriata	Nissum3	AF417652	Lundholm et al. 2003
Nitzschia pellucida	99NG1-16	AF417672	Lundholm et al. 2002
Nitzschia laevis	M1285	AF417673	Lundholm et al. 2003

FIGURES

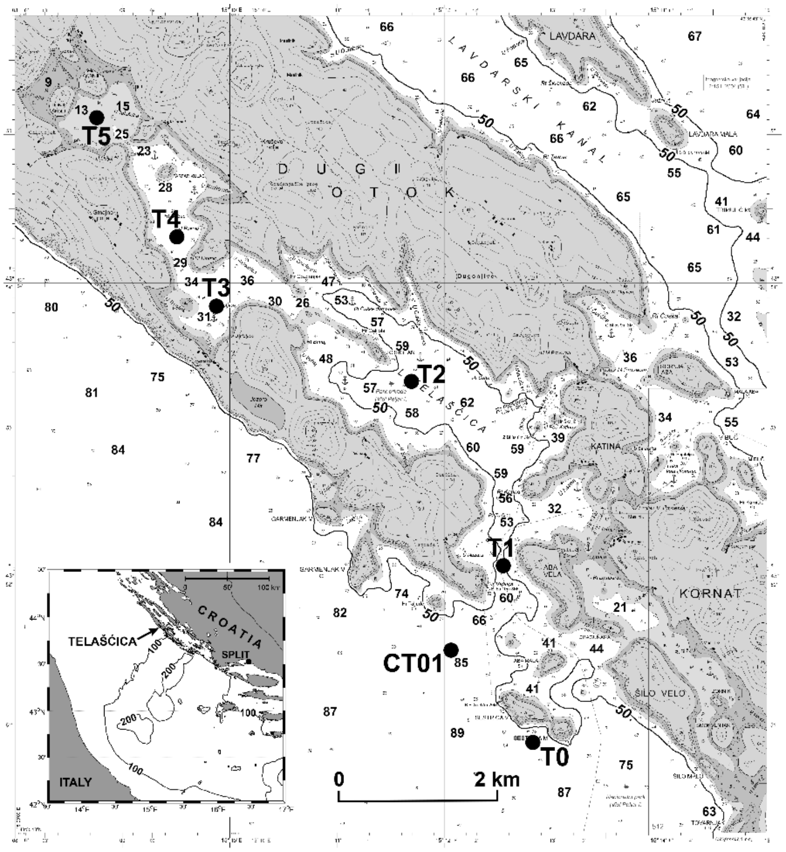

Figure S1. Telašćica Bay; sampling area. T0 is located on the southern side of the bay with the depth of 85 m and it is a referent site without any anthropogenic influence. Additional station (CT01) close to T0 was investigated in March 2012, to get better insight into a physico-chemical conditions. Station T1 is located at the entrance to the bay (bottom depth 55 m) while sampling sites T2 – T5 are situated within the bay and their depth varies between 20 and 60 meters. Characteristic depths in the Telašćica Bay and in the surrounding area are also denoted.

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Publication IV

Blue Diatoms: Global Phenomenon of ″Greening″ in Shellfish and Record of Planktonic *Haslea* Species in the South Adriatic Sea

Plave dijatomeje: globalni fenomen ″zelenih″ školjkaša i nalaz planktonske dijatomeje roda Haslea u južnom Jadranskom moru

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DOI 10.17818/NM/2017/1.7
UDK 639.41 (262.3-13)
Review / *Pregledni rad*
Rukopis primljen / *Paper accepted*: 25. 11. 2016.

Summary

Diatoms are unicellular, photoautotrophic eukaryotic microorganisms, often forming colonies and can be found in most aquatic and moist terrestrial habitats. All known diatoms today have specific golden-brown pigment fucoxanthin that masks chlorophylls in diatom plastid, but one genus represents an exception, having the additional specific pigment marennine due to whom the cells appear blue. Blue diatoms from genus *Haslea* cause a global phenomenon of ˝greening˝ in shellfish (mostly oysters) affecting them in both positive and negative ways. In this study, historical and recent review regarding blue diatoms and physiological and behavioural effect of marennine as well as challenges in shellfish farming from diatom perspective is addressed. This study is also a first record of blue *Haslea* diatom in the South Adriatic Sea during BIOTA (Bio-tracing Adriatic Water Masses) cruise in March 2016. Investigated blue diatom was cultured in laboratory and morphologically analysed with light microscopy. Diatom investigations are very important for better understanding of the ecology of specific marine area, but also for the economy, aquaculture and tourism. The emergence of green coloured flesh of shellfish in the Adriatic Sea has not been recorded yet, but this finding of the blue diatom from genus *Haslea* does not rule out this possibility in the future.

KEY WORDS
blue diatoms
marennine
shellfish
Haslea
South Adriatic Sea

Sažetak

Dijatomeje su jednostanični, često kolonijalni, fotoautotrofni, eukariotski mikroorganizmi koje nalazimo u gotovo svim vodenim i vlažnim kopnenim staništima. Većina poznatih dijatomeja je karakterističnog zlatno-smeđeg obojenja zbog pigmenta fukoksantina, dok se jedan rod izdvaja jer sadrži još i pigment marenin koji stanice čini plavo obojenima. Plave dijatomeje iz roda Haslea uzrokuju globalni fenomen ˝ozelenjavanja˝ mesa školjkaša (uglavnom kamenica), a sam fenomen ima i pozitivne i negativne utjecaje na školjkaše. U ovom radu prikazan je povijesni i sadašnji pregled plavih dijatomeja te fizioloških i bihevioralnih učinaka marenina na komercijalno važne školjkaše, a uz plave dijatomeje dan je i pregled izazova u uzgoju školjkaša iz općenite perspektive dijatomeja. Ovaj rad je ujedno i prvi nalaz plave dijatomeje roda Haslea u južnom Jadranskom moru tijekom BIOTA (Bio-tracing Adriatic Water Masses) istraživanja u ožujku 2016. godine. Jadranska plava dijatomeja uzgojena je u laboratoriju, a njezina morfologija je analizirana uz pomoć svjetlosnog mikroskopa. Istraživanja dijatomeja su, osim za bolje razumijevanje ekologije specifičnih morskih područja, izuzetno značajna također i za privredu, uzgoj školjkaša i turizam. Pojava zeleno obojenog mesa školjkaša u Jadranskom moru dosad nije zabilježen, no ovaj nalaz plave dijatomeje roda Haslea tu mogućnost u budućnosti ne isključuje.

KLJUČNE RIJEČI
plave dijatomeje
marenin
školjkaši
Haslea
južno Jadransko more

1. BEHIND THE SCENES: DIATOMS / *Zakulisno: dijatomeje*

Plankton includes organisms that are carried by water currents and are distinguished as phytoplankton (photoautotrophic, mixotrophic or heterotrophic organisms), zooplankton and bacterioplankton depending on their trophic preferences. Photoautotrophic phytoplankton includes microscopic algae that use sunlight and CO_2 to perform photosynthesis and create simple sugar molecules (glucose) and O_2 as a by-product. Diatoms (Bacillariophyta) are mostly photoautotrophic organisms that are usually single-celled but can often form colonies. They are usually called golden-brown microalgae

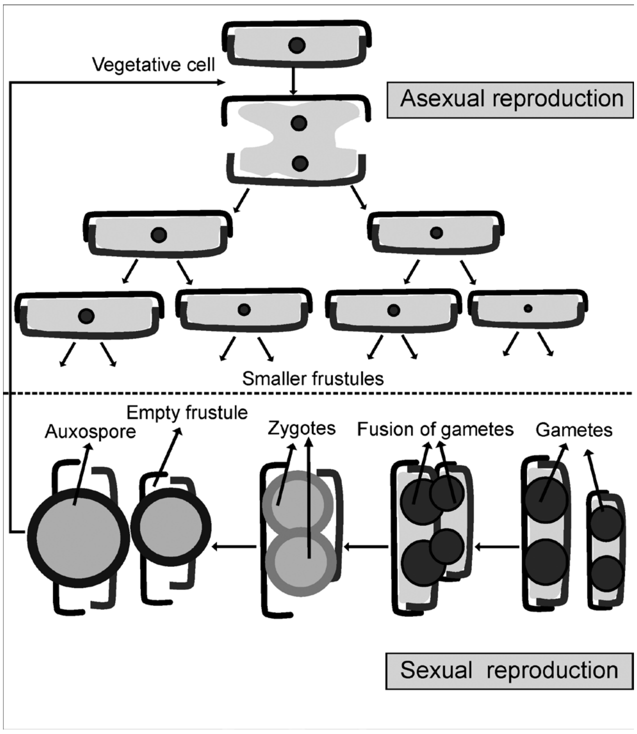

Figure 1 Reproduction in pennate diatoms.
Slika 1. Razmnožavanje penatnih dijatomeja

due to their pigmentation -chlorophyll *a* and *c*, masked by fucoxanthin, diadinoxanthin and diatoxanthin. Concerning phytoplankton, diatoms are most successful group obtaining more than 20% of world's carbon fixation which in total exceeds carbon uptake by rain forests. The unique hallmark of diatoms is the specially silicified cell wall, called frustule, which consists of two halves unequal in size, the epitheca and the hypotheca, that are held in place by silicified girdle bands, and which present a great variety of size and shapes [33]. Diatoms reproduce by mitosis, and when a cell undergoes mitosis, each daughter cell receives one of the two valves of the frustule from the parent cell. The inherited valve is used as the epitheca of the frustule, leaving daughter cell to synthesise its own hypotheca. As a consequence, one daughter cell is identical in size to parental cell, while the other one is smaller, a phenomenon that usually leads to a reduction in the average cell size of the population and to its die-out. After the reduction in the average cell size, diatom cell usually undergoes a sexual phase of reproduction in which zygote turn into auxospore that expands forming the initial cell and restoring the maximal specific cell size (Figure 1). Conventionally, diatoms are divided into two groups based on valve symmetry and their mode of sexual reproduction: centric forms which are radial symmetric and oogamus (i.e. they produce small motile male gametes and large non-motile female gametes) and pennate forms which are boat-shaped bilaterally symmetric and aplanogamus (they do not release flagellate gametes) (Figure 1). Today, taking into account molecular data, we distinguish three major groups of diatoms: Coscinodiscophyceae (radial centrics), Mediophyceae (multipolar centrics) and Bacillariophyceae (pennate diatoms) [21] (Figure 2).

2. GREENING OYSTERS: FINGERPRINT OF BLUE PENNATE DIATOMS / *Zelene kamenice: karakteristični otisak plavih penatnih dijatomeja*

Oysters (Bivalvia, Osteridae), as a shellfish filter feeders, have several important roles by which they help marine ecosystem preserve its balance. Possibly the most important role of oysters is that they are considered to be foundation species of oyster reefs. Also, asoysters are filter feeders, they can greatly influence nutrient cycling in estuarine systems and maintain the stability of the ecosystem. Oysters are economically very important, as a part of mariculture and food industry worldwide.

The first literature record of green oysters dates back to the 17th century when Thomas Sprat in 1667. observed greening of an oyster growing pond near Colchester, England [35]. He also observed that oyster's gills turn green after the pond turned green, and have hypothesised how combined action of sun and earth led to a green colouration of pond sediment. Later on, other studies mentioned more abiotic factors as possible explanations for greening oysters: disease of shellfish similar to obesity [27], ˝liver malfunction˝ [37], oyster ingestion of Priestley's green matter (aggregation of algae firstly described as ˝vegetable˝ responsible for production of oxygen)[38], presence of specific metallic ions in pond sediment, especially copper and zinc [11]. No matter which reason for greening effects took place at that time, green oysters were gastronomically very famous in France and have been celebrated as a delicacy fit to a

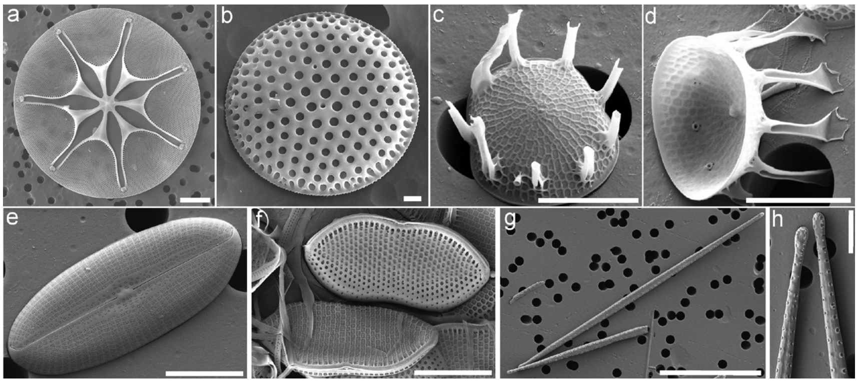

Figure 2 SEM micrographs of three major groups of diatoms: a, b - Coscinodiscophyceae (radial centrics); c, d - Mediophyceae (multipolar centrics); e, f, g, h - Bacillaryophyceae (pennate diatoms). Scale bars: 30 µm (g), 10 µm (a), 5 µm (e, f, h), 3 µm (c, d), 1 µm (b) Photos credit: Carlos E. Wetzel and Sunčica Bosak.

Slika 2. SEM mikrofotografije triju glavnih grupa dijatomeja : a, b - Coscinodiscophyceae (radijalne centrice); c, d - Mediophyceae (multipolarne centrice); e, f, g, h - Bacillaryophyceae (penatne dijatomeje). Mjerilo: 30 µm (g), 10 µm (a), 5 µm (e, f, h), 3 µm (c, d), 1 µm (b). Fotografije izradili: Carlos E. Wetzel i Sunčica Bosak.

king – at the very last for the ˝Sun King˝; it was one of the Louis XIV's favourite meals [18].

First actual experimental work on the green oysters did Benjamin Gaillon, an officer of French Customs during the Restoration and the early July Monarchy [14]. He scraped shells and microscopically observed, as he said, little green motile worms, and consequently, named them *Vibrio ostrearius*. Almost at the same time, controversially, French botanist, explorer and Dragoons cavalry officer during Napoleonic wars – Bory de Saint-Vincent, classified Gaillon's ˝green worms˝ into his ˝psychodaire˝ kingdom which contained all organisms whose position between animal and plant was unclear [3]. He thus proposed a different name for those organisms – *Navicula ostrearia*, because the shape of organisms on the shells reminded him on naviculoid diatoms. After the introduction of electron microscopy in morphological diatom studies, Simonsen transferred the ˝blue navicula˝ from the genus *Navicula* to *Haslea*, a new genus he created for this purpose, based on specific morphological features of the frustule, and he used *Haslea ostrearia* as a type species [34]. *Haslea ostrearia* (Gaillon) Simonsen 1974. is a tychopelagic diatom species, standing for an organism that can be either benthic or epiphytic but also planktonic [33]. *H. ostrearia* is also a euryhaline species (broad tolerance to salinity changes) and can thrive in high light environments (such as shallow ponds exposed to high UV intensity throughout most of the day). One specific physiological feature that distinguishes *H. ostrearia* from other diatoms is the production of *Haslea*-specific pigment called marennine [30]. Marennine is a water-soluble pigment and based on some biophysical and chemical characteristics is possibly a polyphenolic compound existing in two forms; one intracellular and one extracellular, which differ in their spectral characteristics (UV-visible spectrophotometry, Raman spectroscopy) and molecular weight (10.7 and 0.9 kDa, respectively). Based on pH value, marennine can differ in colour from acidic violet-blue to basic green. Cells of *H. ostrearia* actively secret marennine in surrounding water, eventually colouring it in greenish colour, turning shellfish gills and flesh in green (Figure 3). The natural occurring greening phenomenon of oysters besides in England and France was observed in Denmark, United States, Canada and Australia (Moreton Bay, Great Oyster Bay) [16]. Reporting these greening phenomenon, *H. ostrearia* was thought to be a one, cosmopolite species (Figure 4). However, today there are three known blue *Haslea* diatoms: *H. ostrearia*, *H. karadagensis* (Davidovich, Gastineau & Mouget) described from Karadag Natural Reserve, Crimea (Ukraine) and *H. provincialis* Gastineau, Hansen & Mouget described from the area of Boulouris, France [15, 16, 17]. Nevertheless, new blue diatom species from genus *Haslea* are likely to be found worldwide.

3. MARENNINE: APPLICATIONS AND PHYSIOLOGY OF THE BLUE PIGMENT / *Marenin: primjena i fiziologija plavog pigmenta*

Ever since Edwin Ray Lankester in 1886. discovered a new pigment and named it marennine according to Marennes-Oléron area in western France, marennine is being studied in a wide context: as an autotoxin (associated with cell pathological states); as an allelopathy chemical (inhibiting the growth of some algal species encountered in oyster ponds and modifying inter-specific competition among phytoplankton); as antibacterial and antiviral, antioxidant or anti-proliferative agent [20, 26, 30]. A role in the protection against metals such as copper has also been suggested. The exact structure of marennine is still unknown, although it has been hypothesised to be a polyphenolic compound [30]. Additionally, the observation of the ultrastructure of blue cells demonstrated the abundance of vesicles with a diameter of 5 µm in the cytoplasm along with the accumulation of marennine, which suggests that this pigment is synthesised or stored in these vesicles [24]. Marennine plays a significant role in photosynthesis acting as a physical barrier by modifying light quality and intensity as it passes through the water column. On the other hand, global ˝greening˝ phenomenon in shellfish which is a direct consequence of marennine (in most cases the extracellular form of mareninne) has both positive (green shellfish as a delicacy) and negative impact on this important aquaculture branch [32]. Piveteau (1999) demonstrated that oysters fed with *H. ostrearia* in artificial seawater ponds grow slower compared to those fed with the diatom *Skeletonema costatum* over a long period of time (e.g. 8 weeks) [28]. Yet, the reason for the delay of growth in oysters remains unclear; whether it is due to the poor quality of *Haslea* given or biological activity from the marennine released into the ponds. Prasetiya et al. (2015) investigated changes in the clearance rate (CR - volume of water cleared of suspended particles per unit of time) of *Crasostrea gigas* when is fed with cells of *H. ostrearia* and extracellular form of marennine, and concluded that CR significantly decreases (51 %) when compared to control cell suspension without marennine in water [32]. Prasetiya et al. (2016) also showed that two economically important shellfish – *Mytilus edulis* and *Crasostrea virginica* have the behavioural response to higher concentrations of marennine and both species displayed curtailed valve opening compared to control groups that were not exposed to high concentrations of marennine [31]. This is of an extreme importance for shellfish – when valve openings are curtailed, shellfish cannot completely close its shell, leaving it more fragile for predators. Next important effect of marennine on these shellfish is the scope for growth which was 58% lower in *M. edulis* and 85% lower in *C. virginica* under long-term (8 weeks) exposure to marennine [32]. Oxygen uptake in *C. virginica* is also affected by the higher concentration

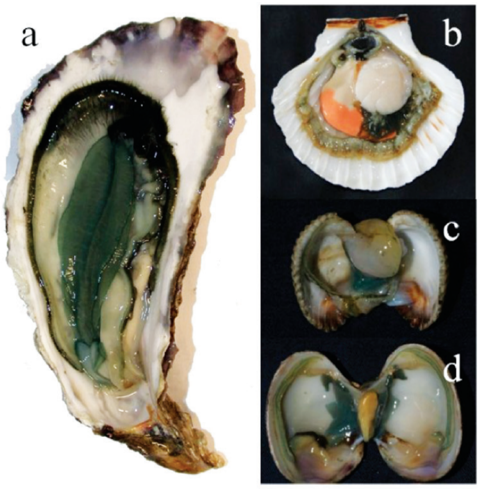

Figure 3 Greening effect of marennine on shellfish. (a) pacific oyster, (b) scallop, (c) cockle, (d) clam. Adapted from Gastineau et al. 2014.

Slika 3. Zeleni efekt marenina na školjkašima a) pacifičkoj kamenici, b) kapici, c) čančici, d) lisanki Prilagođeno iz Gastineau et al. 2014.

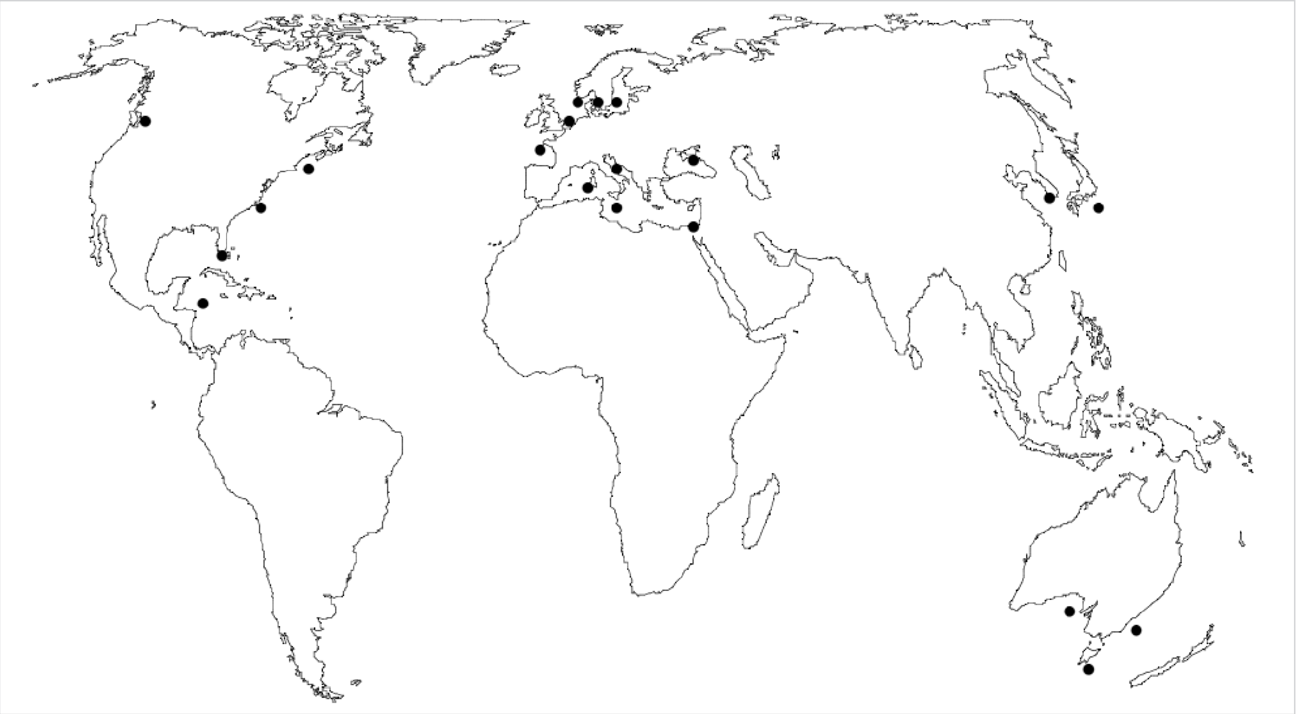

Figure 4 World distribution of *Haslea ostrearia* according to the literature. Each point indicates a site where the presence of *Haslea ostrearia* was assessed from observation of diatom with blue apices, or deduced from the occurrence of green oysters. Map adapted from Gastineau et al. 2014.

Slika 4. Globalno rasprostranjenje dijatomeje Haslea ostrearia prema literaturi. Svaka točka prikazuje mjesto nalaza dijatomeje Haslea ostrearia, direktnim nalazom dijatomje ili posredno nalazovom zeleno obojenih kamenica. Karta preuzeta iz Gastineau et al. 2014.

of marennine - 31.8% lower oxygen uptake while exposed to 2 mg L^{-1} of marennine [32]. Finally, marennine is proved to be negative interactor in the accumulation of energy reserves in shellfish, as both of these economically important species have less ω-3-unsaturated fatty acids accumulated in digestive glands [32]. Future applications of intrinsic blue pigment, marennine, are numerous: food industry (as edible coloration agent), textile industry (as textile or paper paint), antimicrobial or antiviral compound in aquaculture, cosmetic industry (facial lotions with UV protection factor), etc.

4. *HASLEA* IN THE ADRIATIC SEA / *Haslea u Jadranskom moru*

The Adriatic Sea, the northernmost part of the Mediterranean, is a semi-enclosed oligotrophic basin bathymetrically divided into three area: i) the shallow northern Adriatic basin (maximum depth 50 m), ii) central/middle Adriatic basin with depressions up to 280 m and iii) the southern Adriatic basin characterized by a deep Southern Adriatic Pit (SAP) (maximum depth 1230 m). General circulation shaped with two main currents – East Adriatic Current (EAC) which brings highly saline and low-nutrient waters from Ionian and Levantine Seas and Western Adriatic Current (WAC) which carries out large amounts of high-nutrient freshwater from the Po River describes Adriatic Sea as quite heterogeneous marine system with the across-shelf and longitudinal trophic gradient resulting in the asymmetric distribution of the phytoplankton composition, abundance and biomass [29]. In the Adriatic Sea, as well as in world oceans, diatoms are the most abundant counterpart of microphytoplankton. Viličić et al. (2002.) listed 504 species of diatoms in the Adriatic Sea, but we can presume that number is much higher as the discovery of new species happens at a constant rate [40]. Spring phytoplankton bloom is mostly composed of diatoms, especially in the northern Adriatic Sea where they do dominate phytoplankton community all over the year [2]. Due to the oligotrophy of the southern Adriatic Sea, the most abundant primary producers are nanophytoplankton and picophytoplankton, while the higher contribution of diatoms is recorded in closed bays as in Mali Ston Bay or Boka Kotorska Bay [8, 9, 39]. Phytoplankton, especially diatoms are being studied intensively for last two decades in the Adriatic Sea, while genus *Haslea* was documented only two times [23, 25]. Munda (2005.) recorded *H. ostrearia* in the northern Adriatic Sea (Trieste, Italy) during summer period (July and August) and labelled the species as abundant/common on permanent concrete plates that were permanently exposed to fouling, and rare/extremely rare on concrete plates that were scraped and sampled monthly [23]. In the middle Adriatic Sea, *Haslea* spp. was reported on various substrates such as iron, painted iron, wood and concrete [25].

In order to determine bio traces of the Adriatic Sea Water masses and optimise a method of their fast detection, two winter cruises (February/March 2015. and March 2016.) with research vessel ˝Naše more˝ were performed in the South Adriatic Sea. Water samples were collected at selected stations (transect from Dubrovnik to isobath of 1000 m) with 5 L Niskin bottles and phytoplankton net at depths determined *in situ* based on respective CTD profile (Figure 5). Whole phytoplankton community was analysed quantitatively with Utermöhl method. In both years the microphytoplankton community was largely dominated by diatoms, with maximal abundances slightly higher in 2016 than in 2015, 1.8. × $10^{4,}$ cells L^{-1} and 1.2 × 10^4 cells L^{-1}, respectively. In 2015 we observed a somewhat unusual number of diatom cells at greater depths, up to 500 m, probably

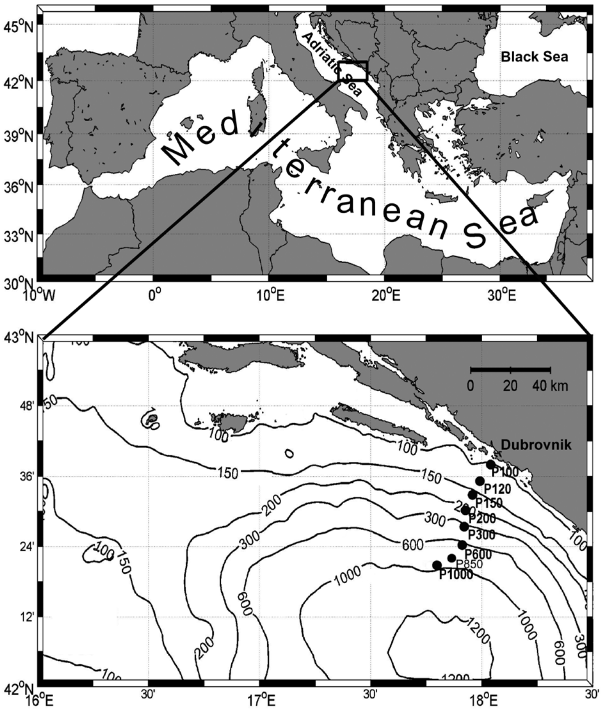

Figure 5 Map of investigated area with stations.
Slika 5. Karta istraživanog područja sa postajama

due to the phenomenon of vertical convection, while the following year the diatoms were only present in the euphotic layer [5]. The finding of particular blue *Haslea* species occurred only in 2016. despite the fact that genus *Haslea* represented by other species was documented in both years.

In addition to standard phytoplankton methods, we took live net samples for establishing diatom laboratory cultures. Immediately upon an arrival in the laboratory, samples were inoculated in Guillard's f/2 Marine Water Enrichment Solution (Sigma-Aldrich, United Kingdom), and after acclimatisation, individual cells were manually isolated in monoclonal cultures. Cells of *Haslea* sp. were at first recognised as belonging to genus *Navicula* due to the naviculoid shape of frustules, but after two weeks of growth in monoclonal culture, the cultures appeared blue-green in colour. Investigated *Haslea* clone BIOTAII-43 was isolated from coastal station P150 (42°32'N; 17°59'E). After more detailed microscopic observation, it was observed that cells in culture have blue apices indicating genus *Haslea* and synthesis of marennine (Figure 6). During a frequent examination of Adriatic *Haslea* sp. cultures, it was observed that with the maturation of cells more marennine was synthesised and accumulated at cells apices. Additionally, aggregation of cells in blue-green floccules appeared at the bottom of the flasks approx. every two weeks. In order to identify Adriatic clone, further morphological examinations were done: light microscopy on live and cleaned diatom material. Cleaning of diatom frustules from organic matter is crucial for the morphological examination due to the necessity of measuring and observing different morphological features of the frustule (i.e. striae, areolae, raphe, central and apical nodes, helictoglossa…). The samples were firstly rinsed with distilled water, followed at the addition of the equal amount (approx. 5 mL) of saturated $KMnO_4$ (or diluted 50%) for oxidation of organic matter and left for 24 hours. The next day an equal amount of concentrated HCl was added, gently heated over a flame and then rinsed again with distilled water five times. Permanent slides were prepared by drying cleaned material on coverslips and mounting in Naphrax. Light microscopy observations were performed with Zeiss AxioVert 200 inverted microscope equipped with DIC and phase contrast (for cleaned material) and Olympus BX51 light microscope (for live material). Minimally 30 frustules were examined for morphometric analyses. The average cell length and width of Adriatic *Haslea* sp. were 89.60 ±1.05 µm and 11.23 ± 0.3 µm, respectively. Compared with other *Haslea* species, Adriatic *Haslea* sp. is larger, as *H. ostrearia* (the largest recorded blue diatom) is 71.8 ± 1.7 µm long and 7.3 ± 0.1 µm wide. Other two blue diatoms – *H. karadagensis* and *H. provincialis* are also smaller than Adriatic *Haslea* sp. - 52.5 ± 0.1 µm and 65.8 ± 0.1 µm in length and 8.0 ± 0.03 µm and 7.4 ± 0.1 µm in width, respectively [17]. After all morphological analyses, the Adriatic clone of genus *Haslea* could only be identified to the genus as *Haslea* sp. (Figure 6). More detailed morphological studies (examination with scanning electron microscopy) and molecular analyses are needed for identification to the species level.

We can hypothesise why blue *Haslea* species haven't been reported in the South Adriatic Sea yet: use of phase-contrast in light microscopy (specific marennine colour remains unrecognised), low number of *Haslea* diatoms in field samples, sampled cells not mature enough to start producing marennine, etc. Giving in mind that previous documentations of *Haslea* in the Adriatic Sea were from northern and middle Adriatic Sea, this study is even more valuable as this is the first record of *Haslea* sp. in the South Adriatic Sea. Studies regarding marine phytoplankton, especially diatoms are important for better understanding of ecosystem in general. Diatoms are good bio-indicators that can inform us about the trophic state

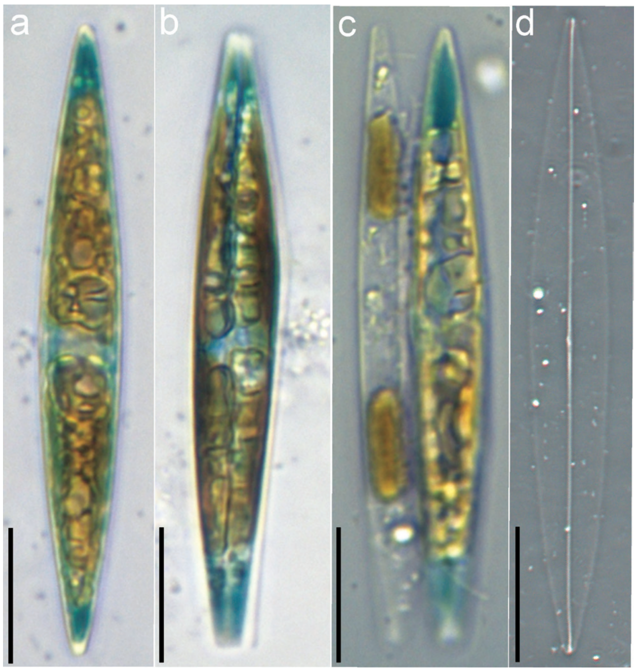

Figure 6 Light micrographs of the Adriatic clone BIOTAII-43 *Haslea* sp. a, b, c - live cells with marennine; b, c – cells in vegetative division; d – light micrograph of cleaned *Haslea* sp. frustule. Scale bar = 20 µm

Slika 6. Svjetlosne mikrofotografije Jadranskog klona BIOTAII-43 Haslea sp. a, b, c - žive stanice s mareninom b, c –stanice u vegetativnom dijeljenju; d – svjetlosna mikrofotografija očišćene Haslea sp. frustule. Mjerilo = 20 µm of the environment, primary production rate, and indirectly, enable estimation of ecosystem sustainability. Understanding the link between primary producers (e.g. diatoms) and higher trophic levels (e.g. shellfish) can generate better economic and environmental outcome. At last, from the scientific point of view, studying diatoms is of an extreme importance worldwide, as these organisms have a high impact on carbon fixation and carbon injection in deeper oceans, which is today burning question of climate change that we all want to reduce.

5. CHALLENGES IN SHELLFISH FARMING FROM DIATOMS PERSPECTIVE / *Izazovi u uzgoju školjkaša iz perspektive dijatomeja (algi kremenjašica)*

Over the last 60 years, world aquaculture production has greatly increased from about 20 million tonnes in 1950. to 150 million tonnes in 2010. while production of marine shellfish presently accounts for 75% of global marine aquaculture [13]. Probably the most important factor that needs to be considered that when defining a link between phytoplankton and filter feeders is that shellfish are non-selective filter feeders, meaning they filter particles just based on their size, not type. Consequently, all shellfish use phytoplankton as their main food source while phytoplankton's primary production and growth depends on various environmental factors such as nutrient availability, light (turbidity) and temperature. This is particularly important as many studies showed when shellfish are grown under similar conditions at various sites; up to 85% of any difference in growth observed can be attributed to water temperature and primary production [19]. Additionally, shellfish can exert ˝top-down˝ grazer control on phytoplankton and in the process of raising turbidity, thereby increase the amount of light reaching the sediment surface and sustain favourable growth conditions for seagrass or benthic algae. In some situations, shellfish can also exert ˝bottom-up˝ control on phytoplankton production by changing nutrient regeneration processes within the sediment [19]. Therefore, continuous monitoring of shellfish farms is necessary to monitor environmental factors such as temperature, oxygen concentrations and nutrient availability and composition and variability of phytoplankton.

In most studies, diatoms have been recognised as the main component of phytoplankton as the available food source for shellfish. An example is the study of Mediterranean Thau Lagoon, where *Ostreococcus tauri*, a minute picoeukaryote (cells smaller than 1µm) is responsible for most of the primary production in the summer, but such small pico particle is not efficiently retained by the gills of shellfish, particularly oysters, so oysters mainly use micro-fraction (diatoms) for food [12]. Generally, diatom species are considered to be a highly nutritious class of microalgae and many species have been shown to promote survival and growth of shellfish [12]. Commercially important *M. edulis* has been shown to capture diatoms from the genus *Phaeodactylum* in preference to smaller and larger natural particles, and similarly, oyster *C. gigas* captures the diatom, *Nitzschia closterium*, preferentially when compared to larger phytoplankton [1, 5]. This can be explained by differences in cell shape or flexibility; in particular, elongated or tri-radiated cells (such as diatoms from genus *Nitzschia* or *Phaeodactylum*) may be more efficiently retained in the shellfish gills than spherical particles of the same volume. Another possibility is that actively swimming cells interact with the ctenidium of some shellfish species in a fundamentally different way. Bricelj et al. (1998) used video endoscopy to study capture and transport of toxic and non-toxic phytoplankton by the ctenidia of two shellfish species [7]. One interesting finding was the difference in how the diatom, *Thalassiosira weissflogii* (11 µm diameter) and toxic and non-toxic strains of the dinoflagellate (*Alexandrium* spp.; 35 µm length) were handled by the ctenidium of *O. edulis* [7]. In contrast to diatoms, dinoflagellates were not retained on the frontal surface of the ctenidium [7]. Not only planktonic diatoms are important food for shellfish - Cognie et al. (2001.) fed four pennate benthic diatom species to the oyster, *C. gigas*, and found that oysters filtered a significantly higher proportion of two intermediate size diatoms (35 – 45 µm length) compared to the smallest (22 µm length) and largest (60 µm length) diatom [12]. This can be particularly important at coastal areas where benthic diatom flora is more diverse than in water column, and therefore can explain preferable ingestion of naviculoid diatoms such as *Haslea* which are in most cases epipelic or epiphytic diatoms.

Shellfish aquaculture in Croatia has more than 50 registered farmers of different shellfish species, but mainly mussels and oysters. Only three breeders have more than 100 tonnes of annual increase, while the rest of them are small farmers, possibly due to the collection of larvae from natural populations to collectors (bypassing larvae controlled production), which has been main breeding method [6]. Regarding only oyster aquaculture, unfortunately it does not even sustain needs for this delicacy during a touristic season (we produce approximately 150 tonnes per year). Most recognisable and prominent nursery on east Adriatic shore is Mali Ston Bay in which oyster farming started a long time ago (the first record is from XVI. century) and where more than 50 farmers are registered [36]. According to the phytoplankton abundance values and phosphate concentrations, the bay has been qualified as moderately/naturally eutrophicated ecosystem [39].

So far, there has been no record of the greening of shellfish in any of the published monitoring programs and studies on shellfish nurseries in the eastern Adriatic Sea. The reason for the lack of data for *Haslea* occurrence could be that *Haslea* species can easily be mistaken with *Navicula* species due to many of morphological similarities. It could be hypothesised that their abundance had never reached values sufficient for the greening effect to take place. Nevertheless, this neglecting of blue *Haslea* diatoms does not need to be amiss since greening phenomenon is not harmful and *Haslea* species are not toxic.

Acknowledgements/*Zahvale*

This study was fully supported by Croatian Science Foundation under the project BIOTA UIP-11-2013-6433. Authors are grateful to Ivna Kavre and Mladen Petravić, Department of Physics and Center for Micro- and Nanosciences and Technologies, University of Rijeka, for help with SEM microscope.

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Publication V

COMPARING METHODS IN PICOPLANKTON ABUNDANCE ESTIMATION

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Abstract

In order to test and compare different methods for picoplankton abundance estimation, a 20-day growth experiment of marine *Picochlorum* sp. was conducted. Cells were harvested daily and its abundance was estimated using three methods (i) counting cells with Birken-Türk haemocytometer, (ii) flow cytometry and (iii) estimation of biomass through Chlorophyll *a* concentrations. Chl *a* concentration showed more similar trend as haemocytometer count suggesting the need for optimisation of each method when higher densities are considered.

Keywords: Analytical methods, Biomass, Chlorophyll-A, Phytoplankton, South Adriatic Sea

Introduction

Picochlorum sp. is a unicellular halotolerant picoalga (Trebouxiophyceae) that has been used multiple times for investigations of its biotechnological properties and potential usage in industry [1]. Importance of marine picoalgae in general have been recognized since their discovery in late 1970's as the "missing link" in the controversial carbon supply since they can contribute greatly to global carbon cycling, biomass and productivity in the sea [2]. Since their importance and challenges in its detection and biomass estimation due its size, we performed a study using *Picochlorum* sp. as model organism to distinguish the best-fit method for accurate estimation of its abundance/biomass during long term cultivation.

Material and Methods

Picochlorum sp. was isolated from south-eastern Adriatic Sea, Croatia and taxonomically identified using nuclear 18S rDNA and chloroplast 16S rDNA phylogeny. Xenic strain PMFPPE4 was used for laboratory growth rate experiment during 20 days. Growth was maintained in Guillard's F2 Marine Water Enrichment Solution (Sigma-Aldrich, United Kingdom) under constant conditions: temperature – 22°C to 22.5°C; light – 30 μmol photons m^{-2} s^{-1} with photoperiod of 16 h of light: 8 h of dark; continuously shaking on Orbital Shaker OR100 (Cole Parmer, UK) at the shaking frequency 80 rpm for 12 h during the day. Starting inoculate of Picochlorum sp. (cca. 10^6 cells mL^{-1}) was established in 200 mL Erlenmeyer flasks in triplicates. Cells were harvested daily and analysed with three different methods. For abundance estimation, cells were examined under inverted light microscope (Olympus BX51TF (Olympuse corporation, Japan) equipped with camera ARTCAM-300MI (Artray Co. Ltd, Japan) and counted using Birken-Türk haemocytometer chamber. Additionally, 1 mL of each triplicate from fresh culture was preserved with 0.1% glutaraldehyde (final conc.), deep frozen in liquid nitrogen, stored at -80°C and analysed with FACSCalibur flow cytometer (Becton Dickinson, San Jose, California). The samples were diluted to approx. same densities with F2 to avoid coincidence, and count was kept below 800 events/s. Number of cells mL^{-1} was then calculated and standard deviation (SD) was included in generating the growth rate graph. For HPLC analysis 1 mL of culture filtered through 0.7-μm-pore-size GF/F filters with syringe and a filter holder (Whatman, United Kingdom) and flesh frozen in liquid nitrogen. Chl *a* concentration was determined by reversed phase HPLC following the protocol of Barlow et al. [3]. Extracts were mixed 1:1 (v/v) with 1 M ammonium acetate and injected into an HPLC system equipped with 3 mm Thermo-Hypersil column MOS2 (C-8, 120 A pore size, 150 × 4.6 mm) (Thermo-Hypersil-Keystone).

Results and Discussion

Growth curve of *Picochlorum* sp. during 20-day experiment is shown in Fig 1. *Picochlorum* sp. showed acclimatization and steady growth during first 7 days of culturing after which entered exponential phase of growth that lasted until day 14. Afterwise stationary phase occurred with day 15, when cells started to aggregate on the bottom of Erlenmeyer flasks. Average daily growth was $1 \times 10^6 \pm 3 \times 10^5$ cells mL^{-1} (haemocytometer counts) and $2 \times 10^6 \pm 4 \times 10^5$ (flow cytometer counts). Average daily abundances in acclimatization ($9 \times 10^6 \pm 6 \times 10^5$), exponential ($3 \times 10^7 \pm 2 \times 10^6$) and stationary ($4 \times 10^7 \pm 2 \times 10^6$) phase according to haemocytometer counts were higher than those counted by flow cytometer: $2 \times 10^6 \pm 5 \times 10^5$ (stationary), $2 \times 10^7 \pm 3 \times 10^6$ (exponential) and $3 \times 10^7 \pm 5 \times 10^6$ (stationary). According to Chl *a* concentrations, daily concentrations in batch cultures during acclimatization phase were 5.84×10^4 ng L^{-1} after which concentrations increased during exponential phase (2.15×10^5 ng L^{-1}), and stabilized (5.14×10^5 ng L^{-1}) in stationary phase. Likewise, large peaks in Chl *a* concentrations after day 12 (as observed in haemocytometer counts) can be explained different behaviour of cells observed in older cultures (i.e. cultures that are in stationary/dying phase). The standard deviation of data obtained from triplicate by flow cytometer counts increases after 12 days, when cell densities are higher. This suggests that in spite of the sample dilution prior to analysis, the abundance counts are more accurate in lower cell densities in this instrument. To conclude, all tested methods give more accurate counts during exponential phase. So, that's not just the method, but the culture growth phase that needs to be considered.

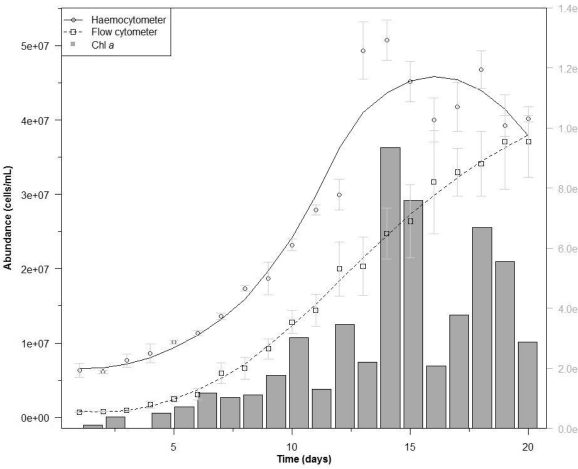

Fig. 1. The growth curve of *Picochlorum* sp. presented through three different methods (haemocytometer, flow cytometer and Chl *a* values) for three replicate cultures.

Acknowledgments

This work has been supported by Croatian Science Foundation under the project BIOTA UIP-11-2013-6433.

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Publication VI

TITLE

Characterization of newly isolated photosynthetic marine pico green algae (*Picochlorum*, Trebouxiophyceae) from the Adriatic Sea

RUNNING TITLE

New *Picochlorum* isolate from the Adriatic Sea

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ABSTRACT:

Photosynthetic picoeukaryotes represent smallest eukaryotic phytoplankton fraction of the world’s seas and oceans, greatly contributing in carbon fluxes, biogeochemical cycles and overall primary production. During BIOTA (Bio-tracing Adriatic Water Masses) cruise in February 2015, strain of unknown photosynthetic pico-green algae PMFPPE4 was isolated in monoclonal culture and subsequent analyses were perfomed in order for correct taxonomical identification and biotechnology potential characterization. Strain PMFPPE4 proved to belong to genus *Picochlorum* (Henly) through morphology (LM and TEM), phylogeny (16S rRNA and 18S rRNA genes) and physiology characterization. Morphology of the strain correlated with other species within *Picochloum* genus, having sphaerical to oval cells from 1.5 to 3.0 µm in diameter, U-shaped chloroplast filling approximately two thirds of the cells and autosporulation in dividing cells. Pigment composition of the strain was congruent with class Trebouxiophyceae, having chlorophyll *a* and *b*, lutein, β-carotene, violaxanthin and neoxanthin, while lipid composition revealed dominant triacylglycerides and membrane glycolipid classes digalactosyldiacylglycerols (DGDG), sulfoquinovosyldiacylglycerols (SQDG), and monogalactosyldiacylglycerols (MGDG), and phospholipid classes phosphatidylcholines (PC), phosphatidylglycerols (PG) and phosphatidylethanolamines (PE). Strain PMFPPE4 showed usual growth rate regime through steady and exponential growth and then stationary phase in a period of three weeks. After that, it was kept in dark for one month after which cells were still viable. Specific lipid structure and survival in dark conditions imply possible switch of this resilient green algae to mixotrophic lifestyle in unflavoured growth conditions. This study represents a first step in resolving the complex taxonomy of this underapreciated and taxonomically unresolved marine genus *Piochlorum*.

KEYWORDS: photosynthetic picoeukaryotes, morphology, phylogeny, physiology, *Picochlorum*, lipids

Introduction

Photosynthetic picoeukaryotes (PPEs) with cell size less than 3 μm represent a significant fraction of the picophytoplankton in the world oceans (Díez et al. 2001). Although PPEs are less numerous than their prokaryotic counterparts, they can greatly contribute to the global carbon cycling in the sea due to their larger volume (Li 1994). Marine PPEs inhabit ocean euphotic zone in concentrations between 10^2 and 10^5 cells mL^{-1} (Li 2009) and, as essential components of microbial food webs, are found to greatly contribute to global biomass and primary productivity in oligotrophic environments (Li 1994). In the marine ecosystem, they are the major contributors to primary production through their capacity to perform oxygenic photosynthesis (Jardillier et al. 2010), and to export and sequester organic carbon to the deep ocean and sediments (Richardson and Jackson 2007). Recently, PPE diversity research has been intensified by applying novel molecular approaches and metabarcoding of environmental DNA (high-throughput sequencing of DNA markers) with mostly variable regions of 18S rRNA gene, allowing better understanding of diversity and importance of the pico-world (de Vargas et al. 2015). We owe today’s vast knowledge to cross-oceanic expeditions such as Malaspina, *Tara* Oceans and Biosope (Duarte 2015; De Vargas et al. 2015; Bork et al. 2015; Claustre et al. 2008; Grob et al. 2007). Most diverse eukaryotic organisms in mentioned expeditions always belong to piconanoplankton (cells ≤ 5μm), among which permanent phototrophs are diatoms, prymnesiophytes, some cryptophytes, haptophytes and chlorophytes and one third of the community is composed from heterotrophic or parasitic dinoflagellates (De Vargas et al. 2015; Shi et al. 2009). Trebouxiophyceae, as a class within the ″core″ chlorophytes, roughly compose one third of the sequences in oceanic temperate areas and are more abundant along the upwelling zones and in nutrient rich coastal zones (Tragin et al. 2016). In the Mediterranean, Diez et al. (2001) and Massana et al. (2004) during PROSOPE cruise have shown a large diversity of PPEs, including putative photosynthetic representatives from a wide range of classes (e.g. Chrysophyceae, Cryptophyceae, Prasinophyceae and Prymnesiophyceae; Vaulot et al. 2008). Nowadays, studies in world oceans focus on using a new molecular marker – plastid 16S rRNA gene, designed to target photosynthetic organisms by using a marine algal-plastid biased PCR primer (Fuller et al. 2006a) as well as PPE class-specific oligonucleotide probes (Fuller et al. 2006b). Thanks to Decelle et al. (2015), we today have an extensive reference database of the plastidial 16S rRNA gene (PhytoREF) including sequences from all major lineages of photosynthetic eukaryotes, comprising terrestrial, freshwater and marine organisms.

Besides molecular approaches to PPE taxonomical assignation, various methods can be used (e.g. microscopy, flow-cytometry, culturing, photosynthetic pigment analysis, physicochemical approaches) (Vaulot et al. 2008). Taxonomic assignment of small coccoid green microalgae is particularly problematic due to the limited number of morphological characteristics and by their minute size (as small as 0.8 μm for the prasinophyte *Ostreococcus*; Vaulot et al. 2008), lack of distinctive morphological features, and fragility when classical fixatives are used (Vaulot et al. 1989). For many years, light microscopy has been most used tool to observe and describe phytoplankton cells; however, for picoplankton cells it is very limited. On the other hand, electron microscopy, such as TEM allowed visualization of important diagnostic features (presence and shape of flagellar hairs or body scales, presence of pyrenoids and starch, chloroplast organisation and membrane configuration (Eikrem and Edvardsen 1999; Ral et al. 2004). Culturing through enrichment cultures, pre-filtered cultures, flow-cytometry sorted or manually isolated and serial diluted techniques allowed scientist to describe and culture various picoplankton species (e. g. *Micromonas pusilla* (Butcher 1952)) that in the end drastically improved our world collections of PPEs (Andersen and Kawachi 2005;Vaulot et al. 2004). At the simplest level, photosynthetic pigments (as the key taxonomic diagnostic feature for microalgae) allows us distinguish green, brown and red algae, but photosynthetic pigment signature is often indicative of the class (e.g. prasinoxanthin is only present in Prasinophyceae) (Guillou et al. 1999). Additionally, physicochemical approaches can be useful in studies of applied biology, such as biotechnology.

Pico-green algae are divided into 16 groups corresponding to classes, orders or recently discovered clades: Pyramimonadales, Mamiellophyceae, Nephroselmidophyceae, Pseudoscourfieldiales, Prasinococcales, Prasinophyte clade VII, Prasinophyte clade VIII, Prasinophyte clade IX, Palmophyllales, Pedinophyceae, Chlorodendrophyceae, Picocystophyceae, Chloropicophyceae and the UTC classes: Ulvophyceae, Trebouxiophyceae and Chlorophyceae that are considered to be ″core″ chlorophytes (Tragin et al. 2016; Dos Santos et al. 2017). Most of these groups have certain morphological attributes and ecological preferences that are unique for species belonging to the respected group (Tragin et al. 2016; Dos Santos et al. 2017). Marine Trebouxiophyceae genera besides *Picochlorum* are few: *Chlorella* (1.5 to 10 µm in diameter), *Elliptochloris* (5 to 10 µm in diameter), *Chloridium* (~15 µm in diameter), *Prasiola* (C.Agardh) Meneghini (in high intertidal zones often associated with guano deposits from seabirds and marine mammals, thalli with multiseriatae stipes), *Brandtia* Hoshina (symbiotic green coccoid in ciliates) and *Phyllosiphon* J.G.Kühn (biofilm associated siphonus parasitic green algae); leaving *Picochlorum* to be unique unicellular pico sized marine trebouxiophyte without similar genera to be mistaken with (Tragin et al. 2016; Motti et al. 2005; Hoshina et al. 2018; Procházková et al. 2015). Hepperle and Krienitz (2001) state the so-called ″*Chlorella*-″ and ″*Nannochloris*-like″ algae that are difficult to identify, i. e. it is difficult to say what a ″real *Chlorella*″ and ″real *Nannochloris*″ is. With molecular support (18S rDNA phylogeny), Henley et al. (2004) managed to move 13 marine/saline isolates from ″*Nannochloris*-like″ algae into *Picochlorum* gen. nov. W.J.Henley, J.L.Hironaka, L.Guillou, M.A.Buchheim, J.A.Buchheim, M.W.Fawley & K.P.Fawley. Today, genus *Picochlorum* summarizes 5 species belonging to PPE of which 3 are taxonomically accepted: *P. oklahomense* Hironaka (as a type species), *P. maculatum* (Butcher) Henley, Hironaka, Guillou, M.Buchheim, J.Buchheim, M.Fawley & K.Fawley and *P. atomus* (Butcher) Henley, Hironaka, Guillou, M.Buchheim, J.Buchheim, M.Fawley & K.Fawley.

Aim of this study is to further improve knowledge and to taxonomically identify newly isolated strain PMFPPE4 isolated from the oligotrophic southern Adriatic Sea through morphological analysis (using light and transmission electron microscopy), phylogeny (using 16S rRNA and 18S rRNA genes) and physiological characterization with pigment and lipid composition within cells.

Materials and methods

Culture establishment and growth rate experiment

Sample containing harvested cells of unknown pico trebouxiophyte was taken during February/March 2015 (28 February – 3 March 2015) BIOTA (Bio-tracing Adriatic Water Masses) cruise conducted in southestern Adriatic Sea. Seawater sample was collected at station

M300 (42.48 N; 17.28 E) using phytoplankton net with pore size 20µm. Immediately upon arrival in the laboratory, the sample was inoculated in Guillard's f/2 Marine Water Enrichment Solution (Sigma-Aldrich, United Kingdom). After mixed culture had grown, unknown PPE cells were filtered into a fresh medium through 3.0-µm-pore-size Nucleopore polycarbonate membrane filters (Whatman, United Kingdom) with a syringe and filter holder. After filtering, isolation continued by the dilution method which consisted of transferring, in a repeated fashion, a sub-volume of a culture (1/10 of the medium volume) to a fresh medium (9/10 of the medium volume), in order to obtain statistically one cell per tube at the end of the series (Knight-Jones 1951; Throndsen 1978). Xenic culture of strain PMFPPE4 was established and it was subsequently transplated every two weeks with fresh medium, always keeping the cells in exponential phase of growth. Strain PMFPPE4 is available at University of Zagreb, Faculty of Science, Laboratory for Biological Oceanography, from M.M. upon request.

The growth rate experiment lasted for 20 days through which PMFPPE4 culture was grown in the following conditions: temperature from 22°C to 22.5°C; light 30 µmol photons m^{-2} s^{-1} with photoperiod of 16 h of light: 8 h of dark; continuously shaking on Orbital Shaker OR100 (Cole Parmer, UK) for 12 h during the light period. Starting inoculate of cca. 10^6 cells mL^{-1} was established in 200 mL of f/2 medium in Erlenmeyer flasks in triplicates. Cells were counted in triplicates daily using Birken-Türk haemocytometer. Number of cells mL^{-1} was then calculated and standard deviation (SD) was included in generating the growth rate graph. Graph was plotted using R software (version 3.4.3) (R Development Core Team 2008) using the ″ggplot2″ package (Wickham 2009).

Pigment and lipid analysis

In addition to the cell counts, pigment analysis with high-performance liquid cromatography (HPLC) was made daily. 1mL of culture in exponential phase of growth was filtered through 0.7-µm-pore-size GF/F filters (Whatman, United Kingdom) and fresh frozen in liquid nitrogen. The extraction in 4 mL of cold 90% acetone was performed by sonication, and the extract was clarified by centrifugation. The pigments were separated by a reversed phase HPLC following the protocol of Barlow et al. (1997) according to the protocol in Šilović et al. (2011).

For the lipid class determination, 50 mL of PMFPPE4 culture in the stationary growth phase were filtered through the GF/F filters. The filters were stored for three days at -80°C before lipid extraction. The particulate lipids were extracted by a modified one–phase solvent mixture of dichloromethane–methanol–water (Bligh and Dyer 1959). N–nonadecanone (KET) was added as an internal standard to each sample, in order to estimate the recoveries in the subsequent steps of the sample analysis. The extracts were evaporated to dryness under nitrogen atmosphere and re-dissolved in 24 μL dichloromethane. Lipid classes were determined by thin–layer chromatography–flame ionization detection (TLC–FID; Iatroscan MK–VI, Iatron, Japan). Eighteen lipid classes, which constitute total lipids, were separated on Chroma rods SIII and quantified by an external calibration with standard lipid mixture, with a hydrogen flow of 160 mL min-1 and air flow of 2000 mL min^{-1}. Quantified lipid classes include hydrocarbons (HC), lipid degradation indices (fatty acid methyl esters (ME), free fatty acids (FFA), alcohols (ALC), 1,3-diacylglycerols (1,3DG), 1,2-diacylglycerols (1,2DG) and monoacylglycerols (MG)), wax and steryl esters (WE/SE, furtheron discussed as SE as in the phytoplankton monocultures WE are not supposed to be present as WE represent zooplankton storage lipids ((Kattner, 1989)), phytoplankton energy reserves (triacylglycerols (TG)), membrane lipids including three phospholipids (phosphatidylglycerols (PG), phosphatidylethanolamines (PE) and phosphatidylcholines (PC)), glycolipids (sulfoquinovosyldiacylglycerols (SQDG), monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG)), sterols (ST) and pigments (PIG). For this work, we did not take into discussion lipid degradation indices and as well hydrocarbons. The standard deviation determined from duplicate runs accounted for 1–8% of the relative abundance of lipid classes. Total lipid concentrations were obtained by summing all lipid classes quantified by TLC-FID. A detailed description of the procedure is described in Gašparović et al. (2015; 2017).

Morphology

The fresh culture samples were investigated under Olympus BX51TF (Olympuse corporation, Japan) inverted microscope equipped with the ARTCAM-300MI camera (Artray Co. Ltd, Japan). Before examination, PMFPPE4 cells were allowed to sediment onto glass slide for 20 min. The cells were examined using 100× Oil Plane 1.25 objective.

Additionally, for ultrastructural studies by transmission electron microscopy (TEM), cultured cells were fixed in 1% (w/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) for 30 min at 5°C and pelleted by centrifugation at 500 × g for 5 min. Cells were re-suspended with ice cold 50 mM cacodylate buffer (pH 7.2) and embedded in 2% agarose. The agarose with the cells was cut in small pieces and washed twice with ice cold 50 mM cacodylatebuffer (pH 7.2). The cells were then post-fixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C, followed by 10 min wash in ice-cold water. After dehydration in the graded series of ethanol, the material was placed in absolute ethanol overnight. The next day, the material was placed in the mixture absolute ethanol and 100% acetone for 30 min and then in 100% acetone for further 30 min. Afterwords, the material was placed in a mixture of Spurr's medium and acetone, first in ⅓ Spurr's and ⅔ acetone for 30 min, then ½ Spurr's and ½ acetone for 30 min, ⅔ Spurr's and ⅓ acetone for 30 min. This was followed by placing the material in Spurr's medium for 2 hours at 45°C. Finally the material was placed in a plastic mould and polymerized in Spurr's medium at 65°C for 48 hours. Ultrathin sections were made by ultra-microtome Leica Ultracut R and stained with 4% aqueous uranyl acetate for 10 min, then with lead citrate, pH 12.0 for 10 min (Reynolds, 1963). Ultrathin sections were examined using a FEI Morgagni 268D transmission electron microscope (Eindhoven, The Netherland) at 70kV.

DNA extraction and PCR amplification

For DNA extraction, 50 mL of PMFPPE4 culture in exponential growth phase was used and extraction was performed with DNeasy Plant Mini Kit (Qiagen) according to manufacturer’s instructions. The purity of the extracted DNA was assessed with the NanoDrop spectrophotometer (BioSpec-nano (Shimadzu)). The plastid 16S rRNA and 18S rRNA genes were amplified by the PCR. For 16S rRNA gene we used algal plastid biased primer PLA491F (5’-GAGGAATAAGCATCGGCTAA-3’) (Fuller et al. 2006a) as forward and OXY1313R (5’-CTTCAYGYAGGCGAGTTGCAGC-3’) (West et al. 2001) as reverse, while for 18S rRNA genewe used Euk63F (5’-CGCTTGTCTCAAAGATTA-3’) as forward and Euk1818R (5’-ACGGAAACCTTGTTACGA-3’) as reverse primer (Lepère et al. 2011).

PCR mixture for 16S rRNA gene (50µL) contained 10 µL 1 × GoTag® Flexi green Buffer (Promega), 2.5 µL magnesium chloride (1.25 mM MgCl2, Promega), 1 µL dNTP mix (1.25 mM, Promega), 2.5 µL of each of the primers (10 µM), 0.25 µL GoTaq® DNA polymerase (100 U, Promega) and 3 µL of template DNA. For 16S rRNAgene PCR was performed with the following conditions: initial denaturation at 95°C for 5 min; followed by 40 cycles at 95°C for 45 s, 60°C for 45 s,72°C for 1.15 min; and final extension at 72°C for 7 min. For 18S rRNAgene PCR mixture (50µL) contained EmeraldAmpMax PCR Master Mix© (Takara Bio, USA) in volume of 25 µL, miliQ H20 (17 µL), 2 µL of each of the primers (10 µM) and 4 µL of template DNA. The PCR reaction was performed with the following conditions: initial denaturation at 98°C for 30 s; followed by 35 cycles at 98°C for 10 s, 55°C for 30 s and 72°C for 1.00 min; and final extension at 72°C for 10 min. The PCR products were quality-assessed on agarose gels prior to purification with the StartaPrep PCR Purification Kit (Agilent

Technologies, Inc.). The purified products were sent for Sanger sequencing (Macrogen, The Netherlands).

Sequence processing, multiple sequence alignment and phylogeny

Partial sequences of 16S rRNA and 18S rRNA genes were checked, edited and paired (5’–3’ and 3’–5’ ends) using Sequencher 4.1.4 (Gene Code Corporation, U.S.A.). Blast analysis was done for all sequences with blastn tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences of strain PMFPPE4 are deposited in GenBank under accession numbers KU843868.1 for 16S rRNA gene and MH010869 for 18S rRNA gene.

A total of 20 taxa for each gene were included in the phylogenetic analyses (Table 1). Outgroup in 16S rRNA gene dataset included four sequences belogning to marine coccoid prasinophyte *Pycnococcus provasolii* and *Pycnococcus* sp. while outgroup in 18S rRNA gene dataset included freshwater autosporulating *Marvania coccoides* and *Marvania geminata*. Multiple sequence alignments were performed in AliView ver. 1.18 with Muscle algorithm under default parameters (Larsson 2014). Alignments were checked with eyeball and no sites were manually excluded. Alignments are available at zenodo link: https://zenodo.org/deposit/1186231.

Table 1. List of all taxa used for phylogeny inference in this study with culture ID, location from which strains were isolated (if applicable) and accession numbers for two different genes. Bold text used for new submitted sequences. n/a – information not available

Taxon	Culture ID	Location	18S rRNA gene	16S rRNA gene
Picochlorum sp.	PMFPPE4	South Adriatic Sea,	**MH010869**	**KU843868.1**
Picochlorum sp.	RCC13	Pacific Ocean	KT860853.1	AY702135.1
Picochlorum sp.	RCC9	Mediterranean Sea	KT860852.1	AY702134.1
Picochlorum sp.	RCC14	Atlantic Ocean	KT860854.1	
Picochlorum sp.	RCC289	Pacific Ocean	KT860649.1	AY702148.1
Picochlorum sp.	RCC475	Mediterranean Sea	KT860662.1	LN735427.1
Picochlorum sp.	RCC1034	Pacific Ocean		LN735208.2
Picochlorum sp.	RCC846	n/a	KT860820.1	LN735467.3
Picochlorum sp.	RCC945	n/a	EU106791.1	LN735497.3
Picochlorum sp.	KMMCC C-275	Yellow Sea; South Korea: Gunsan	GQ122381.1	
Picochlorum sp.	KMMCC 44	Yellow Sea; South Korea: Busan	JQ315636.1	
Picochlorum sp.	KMMCC C-93	Yellow Sea; South Korea: Busan	GQ122341.1	
Picochlorum oklahomense Hironaka	DHmm4W1	n/a	KU561126.1	
Picochlorum oklahomense Hironaka	Xmm7W6	n/a	KU561200.1	
Picochlorum oklahomense Hironaka	n/a	n/a	AY422073.1	
Picochlorum maculatum (Butcher) Henley, Hironaka, Guillou, M.Buchheim, J.Buchheim, M.Fawley & K.Fawley	DHmm1W1	n/a	KU561115.1	
Picochlorum eukaryotum W.J.Henley, J.L.Hironaka, L.Guillou, M.A.Buchheim, J.A.Buchheim, M.W.Fawley & K.P.Fawley	n/a	n/a	X06425.1	
Koliella planctonica Hindák	n/a	n/a		AF497783.1
Koliella spiculiformis (Vischer) Hindák	n/a	n/a		AF278746.1

Koliella sp.	MDL5-3	USA: Mud Lake, North Dakota	AY352046.1	
Chlorella vulgaris Beijerinck	S706	Turkey: Sinop Sirakaraagaclar Creek		KF981993.1
Chlorella vulgaris Beijerinck	S707	Turkey: Sinop Sarikum Lake		KF981994.1
Chlorella vulgaris Beijerinck	S708	Turkey: Sinop Sarikum Lake		KF981995.1
Chlorella vulgaris Beijerinck	SAG 211.11b	n/a		X13688.1
Chlorella sp.	UMPCCC1222	USA: Marin Lagoon, CA		KM218896.1
Chlorella sp.	UMPCCC1231	USA: Sanibel, FL		KM218898.1
Gloeotila contorta (Lemmermann) Chodat	SAG 41.84	n/a	AY422074.1	
Marvania geminata Hindák	SAG 12.88	n/a	AF124336.1	
Marvania coccoides (Naumann) Henley, Hironaka, Guillou, M.Buchheim, J.Buchheim, M.Fawley & K.Fawley	n/a	n/a	AB080301.1	
Micractinium pusillum Fresenius	SAG 13.81	n/a	AF364101.1	
Tetraselmis sp.	RCC500	n/a		AY702169.1
Pycnococcus provasolii R.R.L.Guillard	CCMP1199	North Atlantic, English Channel		AY702120.1
Pycnococcus sp.	RCC521	Mediterranean Sea		LN735430.3
Pycnococcus sp.	RCC730	Indian Ocean		LN735456.3
Pycnococcus sp.	RCC1495	Pacific Ocean:Japanese coast		LN735262.2

We performed seperate phylogenetic analyses on each gene dataset. First we identified the best model of nucleotide substitution and rate variation across sites using a model selection routine available in the program IQtree v. 1.5.5, with specified command –TESTNEWONLY (Nguyen et al. 2015). Model selection was done using the Bayesian information criterion (BIC) which penalizes for the number of parameters in a model and helps avoid overfitting. We reconstructed phylogenies using maximum likelihood (ML) and Bayesian inference (BI) in IQtree v. 1.5.5 (Nguyen et al. 2015) and MrBayes v. 3.2.6. (Ronquist et al. 2012), respectively. Clade support was assessed using IQtree's UltraFast bootstrap routine (Minh et al. 2013) with 1000 pseudoreplicates specified with –bb 1000 command. Bayesian analyses were carried out with a mixed model strategy, whereby various variants of the Generalised time–reversible model (GTR) were sampled in proportion to their posterior probability (MrBayes option 'nst=mix'). Among–site rate variation in MrBayes was accommodated via a Γ distribution with four rate categories (Γ4) and by estimating the proportion of invariant sites (I). We ran 4 simultaneous Markov chain Monte Carlo (MCMC) simulations, each composed of one cold and three heated chains for a total of 10 million generations with a sampling frequency of one thousand generations. Stationarity and convergence among the MCMC runs was assessed from the MrBayes output (standard deviation of split frequencies and potential scale reduction factor) and by inspecting the posterior distributions in the program Tracer v. 1.6 (Rambaut and Drummond 2007). The first 25% of the sampled posterior distributions were discarded as burn-in.

Results

The newly isolated pico-green algae strain PMFPPE4 cells are green, spherical to oval with a diameter 1.5 – 3.0 μm with U-shaped chloroplast occupying approximately two thirds of the cell (Figure 1A). Cells have smooth surface (Figure 1A and B). No flagella or any kind of cell appendix was observed. All cells divide by autosporulation into two daughter cells with division time of approximately four days. Neither zoospore formation nor sexual reproduction was observed. Revealed by TEM, cells contain one nucleus, one mitochondrion, one lateral U-shaped chloroplast lacking pyrenoide, starch grains mostly present within chloroplast, and no flagella (Figure 1B). HPLC analyses of pigment content revealed chlorophylls *a* and *b*, lutein, β-carotene, violaxanthin and neoxanthin.

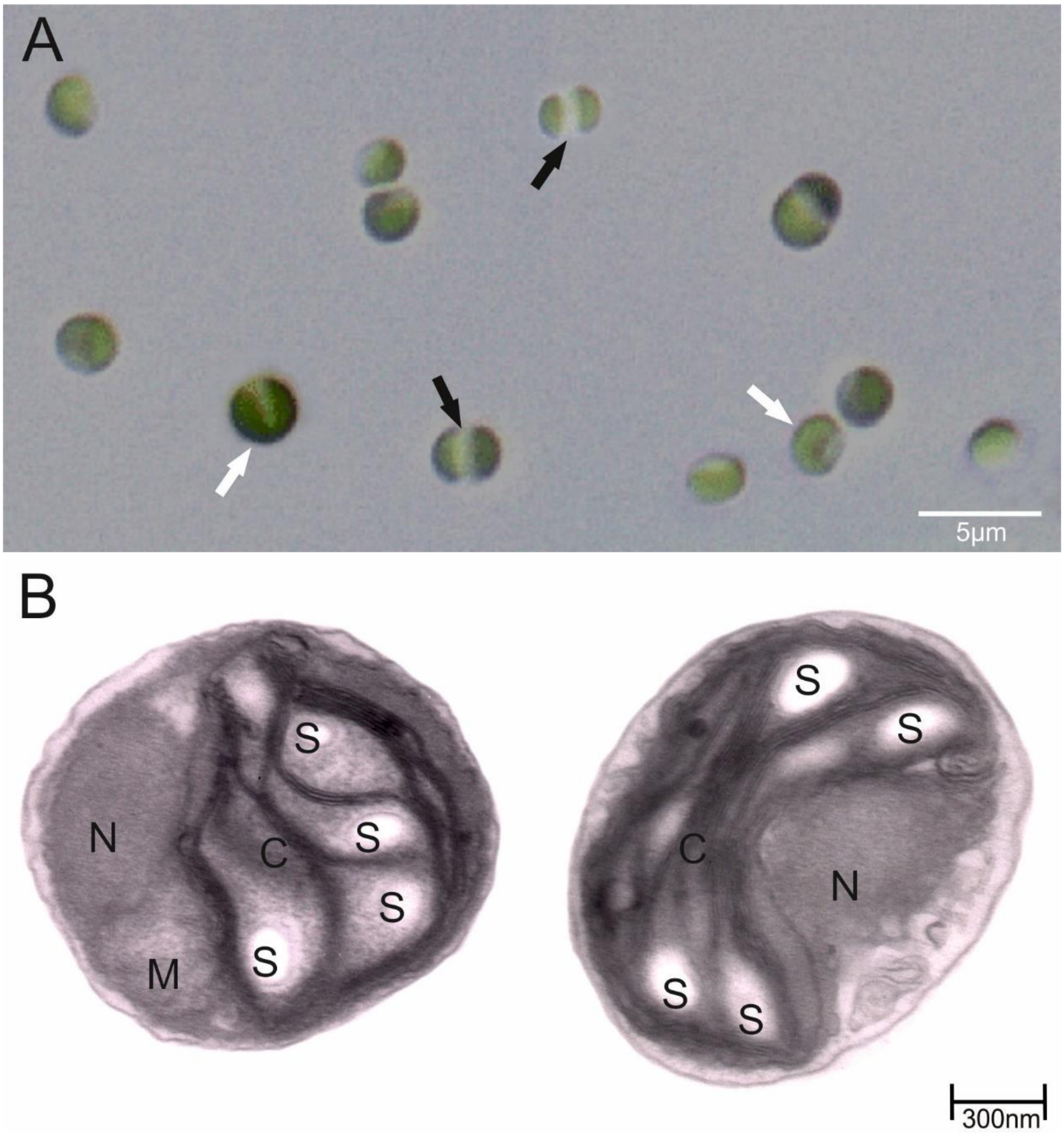

Figure 1. Light (LM) and transmission electron (TEM) micrographs of Picochlorum sp. strain PMFPPE4. A: LM micrographs of strain PMFPPE4. White arrows indicate U-shaped chloroplasts and black arrows indicate dividing autospores. B: TEM micrographs of strain PMFPPE4. N: nucleus; M: mitochondrion; C: chloroplast; S: starch inclusions.

In PMFPPE4 cultures highly considerable amount of particulate lipids, an average of 2256 μg L^{-1} were detected (Figure 2). The most abundant class was triacylglycerols (TG) (17.3%), while among membrane forming lipids the most abundant were glycolipids DGDG (15.8%) and phospholipids PC (12.8%) (Figure 2).

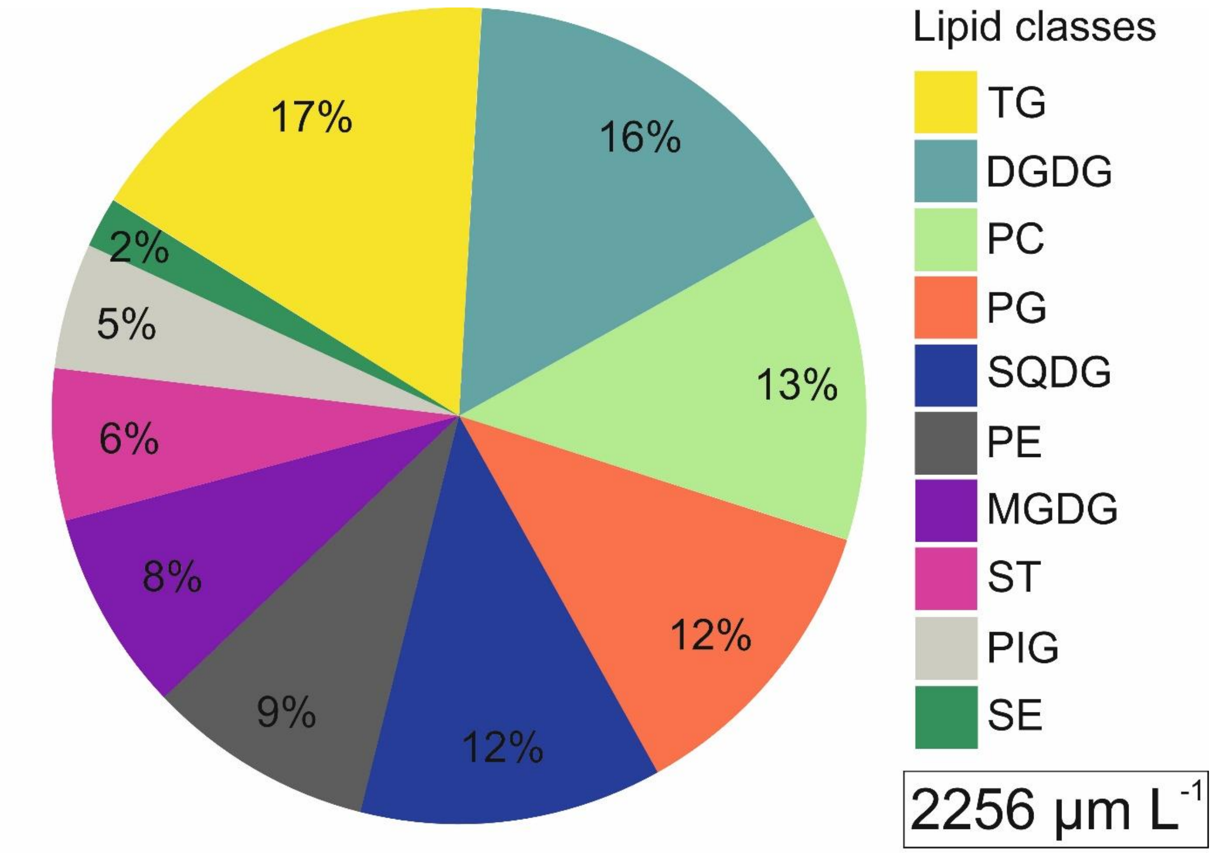

Figure 2. Distribution of PMFPPE4 lipid classes. Total lipid concentrations are given in µg L^{-1} in rectangle, whereas the relative importance are given in % of total lipids.

Cells of the strain PMFPPE4 showed acclimatization and steady growth during first 7 days of the growth experiment with average abundance of 9 × 10^{6} cells mL^{-1} per day. It was followed with exponential phase of growth with average rate of 3 × 10^{7} cells mL^{-1} per day. The largest variation in abundance was between days 12 and 14: 3 × 10^{7} ± 2 × 10^{6} and 5 × 10^{7} ± 2 × 10^{6} cells mL^{-1} respectively. The culture entered in stationary phase two weeks after cultivation, where the cells started to aggregate on the bottom of Erlenmeyer flasks. The abundance stabilized during the last five days of experiment to average values of 4 × 10^{7} ± 2 × 10^{6} cells mL^{-1} (Figure 3).

Due to the extreme resistance of PMFPPE4 in culture conditions and representation of this strain as contaminant and so-called ″weed species″ in many other monoclonal cultures (for example diatoms), additional experiment was conducted. In order to test its physiology, the strain has been kept in dark for one month. After tested period, cells were generally smaller (minimum cell diameter was 1.5 µm) than those grown in 16:8h light: dark periods, but were still viable.

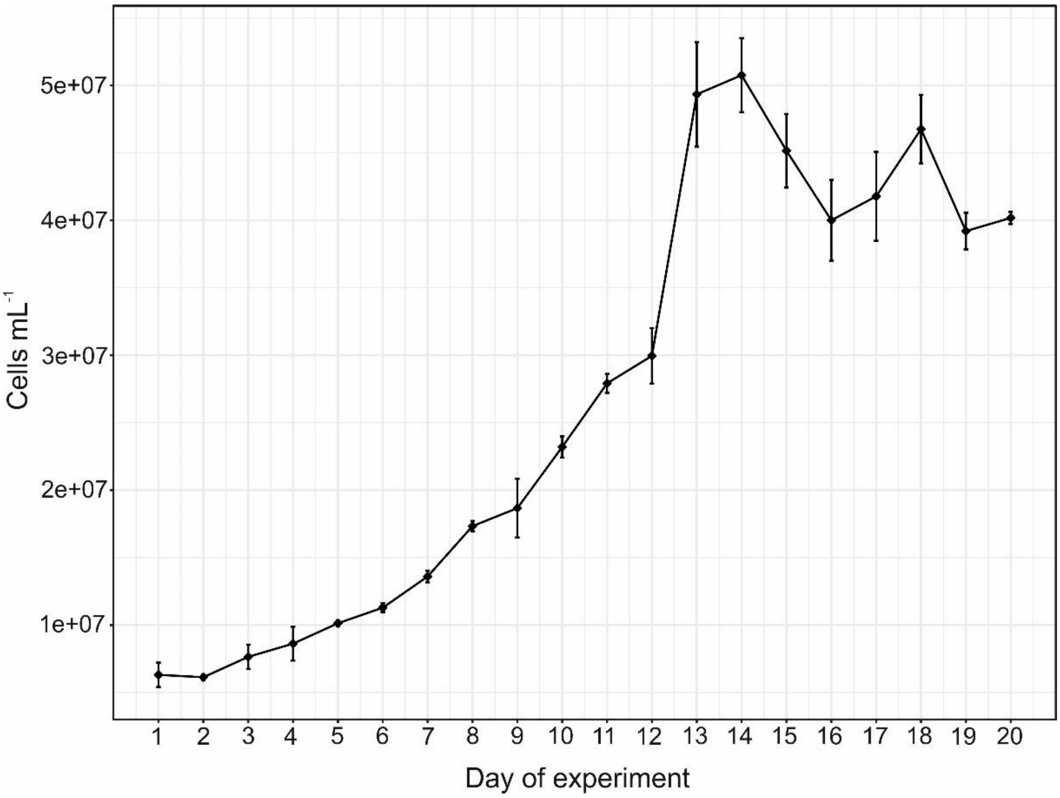

Figure 3. Line graph of average PMFPPE4 cell abundances with standard deviation measured by haemocytometer during over a period of 20 days.

Phylogeny of the 16S rRNA gene confirmed placement of the strain PMFPPE4 into the monophyletic genus *Picochlorum* [Bayesian posterior probability (PP)/Bootstrap proportion (BS), PP/BS=0.93/75 (Figure 4A)], together with other unknown cultured *Picochlorum* species, PP/BS=0.59/80 (Figure 4A). Most similar strains to PMFPPE4 are all from Roscoff Culture Center (RCC), isolated from Pacific Ocean (RCC1034, RCC289 and RCC13) and Mediterranean Sea (RCC9) indicating the cosmopolitan presence of this coccoid pico green algae (Figure 4A). Sister clade containing two more unknown RCC strains of *Picochlorum* sp. (RCC846 and RCC945) divides from PMFPPE4 clade with great support PP/BS=1/99 (Figure 4A). Genus *Chlorella* Beijerinck branches off from monophyletic genus *Picochlorum* with *C. vulgaris* Beijerinck as type species (PP/BS=1/100; Figure 4A) while Koliella Hindák branches off with two species *K. planctonica* Hindák and *K. spiculiformis* (Vischer) Hindák (PP/BS=0.98/85; Figure 4A). With lower support values, *Picochlorum* sp. RCC475, *Chlorella* sp. UMPCCC1222 and *Chlorella* sp. UMPCCC1231 divide from previous clades (PP/BS=0.66/57; Figure 4A). Finally, flagellated unicellular chlorodendrophycean *Tetraselmis* sp. branches off with great support (PP/BS=1/100; Figure 4) and outgroup represented with *Pycnococcus* R.R.L.Guillard clade consisting of *P. provasolii* R.R.L.Guillard and three unindentified *Pycnococcus* species serves as a root of a tree (PP/BS=1/100; Figure 4A).

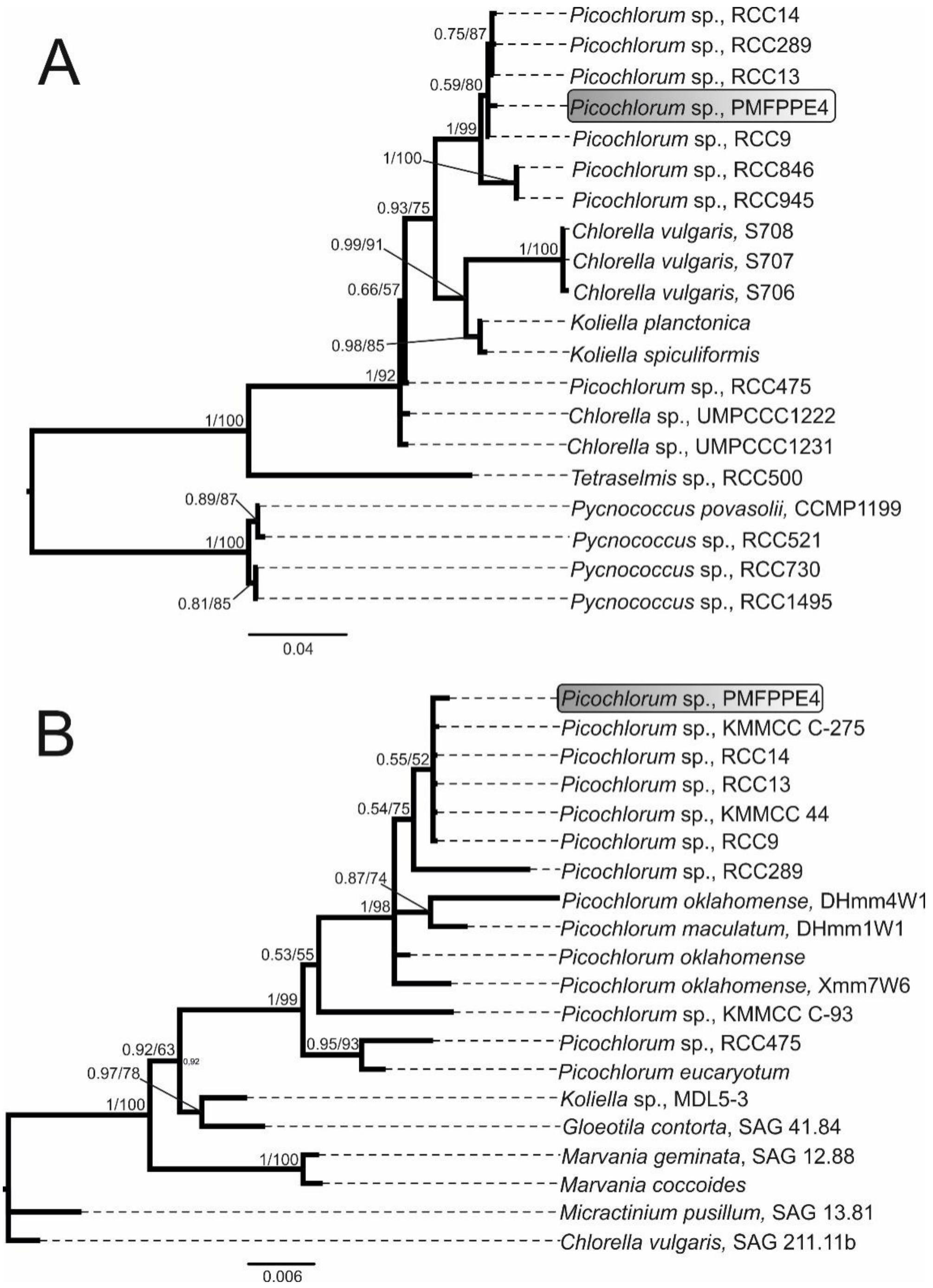

Figure 4. Consensus phylograms inferred with Bayesian inference (BI) and Maximum Likelihood (ML) for 16S rRNA and 18S rRNA gene. Bayesian posterior probability (PP) and Maximum likelihood bootstrap values (BS) over 0.5/50 are indicated above branches. All taxa names consist of genus and species name, then strain if specified in literature. A: 16S rRNA gene consensus phylogram inferred with BI and ML. B: 18S rRNA gene consensus phylogram inferred with BI and ML.

Phylogeny of the 18S rRNA gene also confirmes taxonomic assignation of the strain PMFPPE4 to the monophyletic genus *Picochlorum* (PP/BS=1/99, Figure 4B), grouping it with five other unknown cultured *Picochlorum* strains (PP/BS=055/52; Figure 4B). Besides RCC strains (RCC9, 13 and 14), there are strains KMMCC C-275 and KMMCC 44 isolated from the Yellow Sea (Figure 4B). Here RCC289 branches off PMFPPE4 clade with support PP/BS=0.54/75 (Figure 3B). Generally, genus *Picochlorum* is separated into one big clade (PP/BS=1/98; Figure 4B) consisting of PMFPPE4 clade, separated strain RCC289, smaller clade consisting of *P. oklahomense* strain DHmm4W1 and *P. maculatum* DHmm1W1 (PP/BS=0.87/74; Figure 3B) and separated two more *P. oklahomense* strains (Figure 4B). Next, with lower support (PP/BS=0.53/55; Figure 4B) separates *Picochlorum* sp. strain KMMCC C-93 and with higher support separates clade consisting of *Picochlorum* sp. RCC475 and *P. eucaryotum* (PP/BS=1/99; Figure 4B). Besides genus *Picochlorum*, 18S rRNA gene phylogeny resolved genus *Koliella* and *Gloeotila* Kützing group together (PP/BS=0.97/78; Figure 4B) and genus *Marvania* Hindák with two species *M. geminata* Hindák and *M. coccoides* (Naumann) Henley, Hironaka, Guillou, M.Buchheim, J.Buchheim, M.Fawley & K.Fawley (PP/BS=1/100; Figure 4B). The tree root was consisted from *Micractinium pusillum* Fresenius and *Chlorella vulgaris* (Figure 4B).

DISCUSSION

General morphological characters determined by LM and TEM, along with pigment composition and two gene phylogeneny results undoubtedly identifies strain PMFPPE4 as *Picochlorum* sp. (Henley et al. 2004). Nevertheless, relying on morphologyonly only, this identification would probably be impossible due to inconclusive and few characteristics that can be observed with microscopy (Barcytė et al. 2017). Among the known algal genera, strain PMFPPE4 is morphologically similar to genera *Lunachloris* Baryctė & Hodač or *Neocystis* Hindák by cell shape and parietal chloroplast, but differ with number of divided autospores and lack of mother cell wall around vegetative cells, and neither of the mentioned genera are exclusively marine (Ettl and Gartner 2014; Barcytė et al. 2017). Simmilarities with *Chlorella* and ″*Chlorella*-like″ organisms are high, but species belonging to real *Chlorella* always have pyrenoid present, which is absent in genus *Picochlorum* (Bock et al. 2011). Simmilarities with genus *Nannochloris* Naumann is higher, but this genus appears not to have been taxonomically certain, and without a type species designated (Guiry and Giury 2018). Additionally, several species from genus *Nannochloris* were transferred to *Picochlorum*, indicating there is not enough distinguishable characters that can divide these two genera apart, leaving to taxonomists to deal with the situation of taxonomically appreciateing just genus *Picochlorum* (Henley et al. 2004). Recently, most of the cultures in worldwide culture centers identified as *Nannochloris* sp., were transferred to genus *Picochlorum*, indicating overall taxonomists desire to deal with these two genera in favour to *Picochlorum* (D. Vaulot, personal communication). Examples in defining a genus within chlorophytes on multilayer approach is common (Bock et al. 2011 and references therein). Combination of biochemical characters and molecular phylogeny for example indicate that genus *Chlorella* is polyphyletic, which was previously profoundly refused (Friedl 1995). Physicochemical characteristics of cultured PPE representatives can often help taxonomically positioning certain strains, as Dahmen et al. (2014) showed with identification of *Picochlorum* sp. strain CTM 20019.

We obtained equal positioning of PMFPPE4 within phylogenetic analyses based on two molecular markers (16S rRNA and 18S rRNA genes), which indicate that both of genes serve as specific enough to resolve a phylogenetically placement of chlorophytes on genus level (Tragin et al. 2016; Decelle et al. 2015). In both phylogenies, PMFPPE4 was most similar to other *Picochlorum* sp. strains from RCC, which indicates lack of taxonomical identification within the genus and many possible new, yet undescribed species. Generally, in 16S rRNA gene phylogeny, not one *Picochlorum* sequence is identified to species level, and in 18S rRNA gene phylogeny there are available only three *Picochlorum* species sequences indicating both poor sequence coverage and taxonomical investigations within the genus. Since genus *Picochlorum* is still underappreciated and taxonomically misidentified, most of the strains in cultures are either treated as unknown or are misinterpreted as genus *Nannochloris* (Roscoff Culture Collection RCC, http://roscoff-culture-collection.org/; National Centre for Marine Algae and Microbiota NCMA, https://ncma.bigelow.org/; Culture Collection of Algae at The University of Texas at Austin UTEX, https://utex.org/; The Culture Collection of Algae at the University of Göttingen, Germany SAG, http://sagdb.uni-goettingen.de/). Reasons for such underappreciation of this genus until now is most certainly its minute size, impossibility to identify cells by their auto fluorescence (i.e. flow cytometry), difficult cultivation, and the fact that molecular research on minute coccoid algae started recently (Barcytė et al. 2017). We owe it to molecular phylogeny that many lineages within class Trebouxiophyceae have been discovered recently (Zhang et al. 2008; Eliaš and Neustupa 2009; Neustupa et al. 2009; 2011, 2013a, 2013b; Bock et al. 2010; Fučikova et al. 2014; Song et al. 2015). These findings would still be delayed, if only microscopy or any other limiting technique was applied. Additionally, potentially undescribed microorganisms can be obtained from current field samples, but re-examination of established cultures in available culture collections such as RCC, NCMA or UTEX is of an extreme importance, that would be next step that follows here presented reseach.

Adriatic *Picochlorum* sp. PMFPPE4 strain entered exponential phase of growth between days 7 and 14, and stationary phase after day 15, when cells started to aggregate on the bottom of Erlenmeyer flasks. It takes about 18 days for the *P. oklahomense* cultures to reach maximum biomass concentration in the medium, under the conditions examined by Zhu and Dunford (2013). Additionally, the *P. oklahomense* biomass concentration in the medium started decreasing shortly after it reached the maximum, as it was the case in our study: abundance begun to decrease after day 15, indicating that biomass needs to be harvested as soon as maximum biomass concentration is reached in the medium (Zhu and Dunford 2013).

Ecological preferences of *Picochlorum* sp. PMFPPE4 are yet unexplored, but for Adriatic picoeukaryotes in general, during the same sampling period in 2015, Babić et al. (2017) showed unusual accumulation of PPEs in the layer at the upper border of Levantine Intermediate Water (characterized by higher salinity and lower temperature, abbr. LIW) which was below the euphotic zone. Accumulation of PPEs was not significantly correlated with one of the ecological parameters (e.g. salinity, nutrients or temperature) suggesting that they are not developing in its ecological optimum (Babić et al. 2017). Vertical density gradients were relatively strong in that area, while geostrophic currents indicated a strong vertical shear (Babić et al. 2017). The shear may cause vertical instabilities and transport of water parcels from surface to deep layers and it might be responsible for the occurrence of the PPEs maximum at depths below the euphotic zone. The results from the cruise during which the strain PMFPPE4 was isolated, show a clear fluorescent signal of the PPE maxima at station P600, 280 m of depth, although there was no light available (Babić et al. 2017). According to that, there is also a possibility that the cells switched to mixotrophy (or were mixotrophs from the beginning), providing longer survival in the hostile environment. This indeed can be supported with the fact that the selective pressures on preserving photoautotrophic machinery can be relaxed under certain conditions; such as when the energy costs of maintaining the photosynthetic apparatus outweigh the benefits of its products; or with the fact that picoeukaryotes use phygocytosis in case of mixotrophy/heterotrophy while cyanobacteria, such as prokaryotes, can use only osmotrophy as a specific transport of nutrients from surrounding media (Massana and Logares 2013). This could be mirrored to isolated strain PMFPPE4, were the cells were still viable after a month of being kept in dark. The large lipid content in the cells could also support their availability of surviving in hostile conditions. Dahmen et al. (2014) emphasized biotechnological potential of genus *Picochlorum* and demonstrated its feasibility of using a wild *Picochlorum* sp. as feedstock for aqua-culture and human nutrition or biodiesel production.

Lipid composition of PMFPPE4 culture is in agreement with other algal species but we should be aware that lipid composition of plankton cells varies according to environmental factors (Guschina and Harwood 2009).

The major carbon allocation among lipid classes was TGs that are known as energy storage lipids (Gushina and Harwood 2009). This may indicate on their evolutionary preparation of *Picochlorum* for unfavourable growing conditions. DGDGs together with MGDGs are the major class of lipids presented in the membranes of plastids, which are required not only as bulk constituents of photosynthetic membranes but also for the photosynthetic reaction itself (Kobayashi et al. 2007). High DGDG content, survival in dark conditions and record of photosynthetic picoeukaryotes below photic layer during the same expedition imply possible switch of this resilient green algae to mixotrophic lifestyle in unflavoured growth conditions.

Conclusions

This study identified pico green algae isolated from the southern Adriatic Sea, PMFPPE4, as member of genus *Picochlorum*, underappreciated but widespread genus of Trebouxiophyceae. Assignation of small coccoid algae need to be acomplished through multilayer approach, considering morphology, phylogeny and physiology. This resiliant strain can serve for future biotechnological investigations because of its great potential, as well as long-term maintenance in cultured conditions. Additionally, this study can be considered as a stepping point to *Picochlorum* taxonomical re-establishment using the cultures in available culture collections, with potential new species jet to be described.

Acknowledgments

This work was fully supported by Croatian Science Foundation under the projects BIOTA UIP-11-2013-6433, AMBIOMERES IP-11-2013-8607 and ADMEDPLAN, IP-2014-09-2945. Authors want to thank the crew of RV “Naše more” for their help during the fieldwork. M.M. is thankful to Tanja Vojvoda Zeljko for help during molecular laboratory work.

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Publication VII

Picoplankton winter diversity in an oligotrophic marginal sea

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ABSTRACT:

Autotrophic and heterotrophic picoplankton (cells ≤ 3μm in diameter) abundance and community composition was investigated in the winter of 2016 at three stations along a coast-to-offshore transect in the southern Adriatic Sea. Abundances were estimated by flow cytometry, while community composition through Illumina high-throughput sequencing of 16S and 18S rRNA genes. The photosynthetic picoplankton diversity was also investigated by High-Performance Liquid Chromatography (HPLC) of liposoluble pigments. Heterotrophic bacteria and cyanobacteria (*Prochlorococcus* and *Synechococcus*) accounted for up to 7×10^5; 2.3×10^4 and 2.5×10^4 cells mL^{-1}, respectively, while photosynthetic picoeukaryotes peaked with 3×10^3 cells mL^{-1}. Prokaryotes were dominated by *Alphaproteobacteria* (mainly SAR11, 44.91%), followed by *Gammaproteobacteria* (*Oceanospirillales* and *Pseudomonadales*, 14.96% total), *Bacteroidete*s (mainly *Flavobacteriales*, 13%), *Cyanobacteria* (*Prochlorococcus* and *Synechococcus*, 9.52% total, *Marinimicrobia* (SAR406, 7.97%), *Deltaproteobacteria* (SAR324, 3.83%), *Actinobacteria* (2.24%) and *Chloroflexi* (SAR202, 1.90%). Photosynthetic pigment concentrations were very low (12.12 μgL^{-1} at the most) and taxonomic pigments could be attributed to *Prochlorococcus*, *Synechococcus*, Prymnesiophyceae, Bacillariophyceae, Chrysophyceae and Prasinophyceae. Picoeukaryotes were dominated by heterotrophs, such as Syndiniophyceae, parasitic dinoflagellates (79.67%), Dinophyceae (8.7%) and the radiolarians Collodaria belonging to Sphaerozoidae (22.1%) and Spumellaria (5.0%). The photoautotrophs, including Chlorophyta (Mamiellophyceae, Prasinophyceae, Trebouxiophyceae and Ulvophyceae), Stramenopiles (Bacillariophyta, Chrysophyceae, Dictyochophyceae, Pelagophyceae), photoautotrophic Cryptophyta and some Haptophyta (Prymnesiophyceae), did not exceed 5% of total sequences.

Key words: picoplankton, southern Adriatic Sea, 16S rRNA gene, 18S rRNA gene, High-Throughput Sequencing, diversity, flow cytometry, photosynthetic pigments

INTRODUCTION

Marine picoplankton, unicellular organisms with cell sizes up to 3 μm in Equivalent Sphaerical Diameters (ESD), dominate marine ecosystems both numerically and in terms of carbon biomass, encompassing Archaea, Bacteria, Eukarya (protists and fungi) as well as viruses. Picoplankton are heterotrophic (mainly Archaea and Bacteria) and photosynthetic, the latter including both cyanobacteria (*Prochlorococcus* and *Synechococcus)* and picoeukaryotes (PEs), mainly dominated by prymnesiophytes (Jardillier et al. 2010). PEs include photoautotrophic, mixotrophic and heterotrophic protists, and are an important and mostly overlooked component of marine ecosystems, which under certain conditions can represent the main drivers of plankton ecosystems (as producers, decomposers, parasites, symbionts, etc.) (Li, 1994, Biegala et sl. 2003, Not et al. 2004).

Recent global expeditions such as Malaspina, *Tara* Oceans and Biosope (Pernice et al. 2015; Duarte 2015; De Vargas et al. 2015; Bork et al. 2015; Claustre et al. 2008; Grob et al. 2007) have explored the world’s oceans using genomic approaches and have shed light on the huge and still unknown diversity of marine microbes and of their adaptation to environmental conditions and climate zones. From these dataset, still only partially exploited, it is evident that, among the nanoplankton,microplankton diatoms and dinoflagellates dominate communities worldwide (De Vargas et al. 2015, Shi et al. 2009), with three groups: Alveolata, Rhizaria and Excavata (De Vargas et al. 2015). Instead, dominant photosynthetic picoeukaryotes (PPEs) worldwide are Prymnesiophyceae, Prasinophyceae, Mamiellophyceae, Cryptophyceae, Pelagophyceae, Chrysophyceae and Dictyochophyceae (Shi et al. 2009). Heterotrophic PEs (HPEs) are also very abundant worldwide, defined by high operational taxonomic unit (OTU) numbers (De Vargas et al. 2015). HPEs are mostly bacterivorous (alveolates, stramenopiles), and play a key role in recycling nutrients from the prokaryotic fraction to higher trophic levels (Sherr & Sherr 1994). Likewise, interactions between HPEs and prokaryotes have important ecological implications, as bacterial abundances and community composition are strongly influenced by the predation pressure of HPEs (Jardillier et al. 2005). Besides primary production and bacterivory, HPEs can also influence different trophic levels through parasitic and mutualistic symbiotic associations (Worden & Not, 2008).

On the other hand, bacterial diversity is known in greater details, with SAR11 (*Alphaproteobacteria*) recognized as the most abundant clade everywhere (Fuchs et al. 2005), and clades SAR324 (*Deltaproteobacteria*), SAR202 (*Chloroflexi*) and SAR406 (*Marinimicrobia*) dominating deep-water clades (Guerrero-Feijóo et al. 2017). In the photic zone of oligotrophic areas, the cyanobacteria *Synechochoccus* and *Prochlorococcus* dominate numerically even at depth (Partensky, Hess & Vaulot 1999), with PPEs accounting for 10^2-10^5 cells mL^{-1}, two orders of magnitude lower than cyanobacteria (Li, 2009).

Picoplankton investigations in the Adriatic Sea have mostly focused on *Prochlorococcus*, *Synechococcus*, and total picoeukaryotic spatial and temporal distribution, as revealed by flow cytometry. Bacterial and archaeal community composition in winter mixed conditions has also been investigated by HTS (Korlević et al. 2015; Babić et al. 2018, Šilović et al. 2018 and references therein). Cerino et al. (2012) investigated spatial and temporal variability of pico-, nano- and micro-phytoplankton in the offshore waters of the South Adriatic Sea and confirmed picophytoplankton dominance especially in February through April with cell abundances in the order of 10^7 – 10^8 cells L^{-1}, dominated by *Synechococcus,* with very low contribution by PPEs. Šilović et al. (2011) recorded most PPEs offshore, suggesting a high tolerance for water column instability (Šilović et al. 2011). Najdek et al. (2014) showed that picoplankton community of the South Adriatic Pit (SAP) is strongly affected by 1) intense winter convection episodes in February followed by outbreak of new production in March, and 2) intrusions of highly saline and nutrient-poor Levantine Intermediate Water (LIW). Additionally, vertical mixing injects deep dissolved inorganic nutrients into the upper water column, largely extending the productive layer, and PPEs and *Synechococcus* are documented down to 400 m and 600 m, respectively. For what heterotrophic bacteria are concerned, Korlević et al. (2015) described biodiversity and seasonality of bacterial picoplankton before, during and after deep winter convection in the oligotrophic South Adriatic waters. Most recently, Babić et al. (2018) reported bacterial community composition in the South Adriatic Sea from the surface to the seabed, distinctively describing photic and aphotic bacterial community in unusually semi-stratified winter conditions.

The aim of this paper is to assess the community composition of the whole picoplankton, encompassing archaea, bacteria, cyanobacteria, and picoeukaryotes in the extremely oligotrophic southern Adriatic Sea. The approach used integrated flow cytometry, HTS, and pigment analysis in order to provide a thorough picture of picoplankton diversity and to assess their contribution to ecosystem functioning in the area.

MATERIALS & METHODS

Study site and sampling strategy

The Adriatic Sea is a semi-enclosed oligotrophic basin that can be divided into three areas based upon its bathymetry: i) the shallow northern Adriatic basin (maximum depth 50 m), ii) the central/middle Adriatic basin with depressions up to 280 m and iii) the southern Adriatic basin characterized by the deep South Adriatic Pit (SAP) (maximum depth 1230 m). The basin is characterized by a cyclonic circulation pattern, with re-circulation cells embedded in basin-wide flows, forced by thermohaline features (freshwater influx, wind forces) and geomorphology. This makes the Adriatic Sea an extremely oligotrophic dilution basin – exporting relatively fresh and colder while receiving more saline and warmer waters (Poulain, 2001, Gačić et al. 2001). Two are the main currents – the East Adriatic Current (EAC), which brings highly saline and low-nutrient waters from the Ionian and Levantine seas, and the Western Adriatic Current (WAC), which carries out large amounts of high-nutrients freshwater from the Po River. The South Adriatic Sea is the entering point for the Ionian Surface Water (ISW) and the Levantine Intermediate Water (LIW) and the exit point of the deep water originating from the North. As a consequence of its complexity it is an interesting site to study the interactions between ocean and plankton dynamics.

Samples were collected during the BIOTA winter cruise between 8 and 10 March 2016 at three stations representing coastal (P150, N 42°32′ E 17°59′), mid (P600, N 42°24′ E 17°55′) and offshore (P1000, N 42°20′ E 17°49′) areas (Figure 1).

The physico-chemical and optical properties of the water column during the BIOTA cruise are described in detail in Babić et al. (2018). Briefly, at our stations, seawater temperature, salinity and dissolved oxygen varied between 13.57 - 15.18 °C, 38.68 - 38.90 and 6.32 - 7.37 mg L^{-1}, respectively. Total nutrient concentrations ($NH4^+$, NO_2^-, NO_3^{2-}, PO_4^{3-}, SiO_4^{2-}) were low, therefore indicated extremely oligotrophic conditions in general. NO_2^- and NO_3^{2-} concentrations ranged between 0.062 - 0.138 µM and 0.591 - 1.347µM, respectively. Lowest values of NO_2^- were measured at P600-150m (0.062 µM) and P1000-100m (0.072 µM) while highest values were measured at P150-100m and P150-30m (0.138 - 0.115 µM, respectively). Opposite of NO_2^-, lowest values of NO_3^{2-} were measured at P150-100m (0.591

μM) and P600 - 150m (0.598 μM) while highest values were measured at P600-75m and P1000-100m (1.347 - 1.259 μM, respectively). Phosphates varied between 0.020 - 1.105 μM with maximum measured at P150-100m probably due to mixing of bottom water layer and sediment. Chlorophyll *a* (Chl *a*) was in general very low, with maximum value of 0.45 μg L^{-1} at surface (Babić et al., 2018). Positive values of Apparent Oxygen Utilization (AOU), inversely correlated to Particulate Organic Carbon (POC) suggested that respiration was the main process occurring in the southern Adriatic Sea at the time of sampling (Babić et al., 2018).

Samples for HTS, flow cytometry and pigment analyses were taken at P150 – 30 and 100 m; P600 – 25, 75 and 150 m; P1000 – 80, 100 and 200 m. These depths were chosen based on the vertical distribution of temperature, salinity and Chl *a* fluorescence in order to represent the main water masses distribution at the three stations. Temperature, salinity and Chl *a* fluorescence were measured using a SBE 25 CTD probe (Sea-Bird Electronics Inc., USA) additionally equipped with an SBE 43 sensor for dissolved oxygen concentrations, a WET Labs C-Star transmissiometer for attenuation coefficient and a WET Labs FLNTU for Chl fluorescence and backscattering coefficient (bbp), as reported in Babić et al. (2018).

Seawater for collection of environmental DNA (eDNA) and amplicon sequencing of 18S rRNA gene, for a total of 8L per sample, was pre-filtered through 20 μm pore-size mesh, then through 3 μm-pore-size polycarbonate filters (47 mm Ø, Whatman® plc, UK) in order to select for the pico- fraction, and finally filtered onto 0.2 μm-pore-size polycarbonate filters under low vacuum (47 mm Ø, Whatman® plc, UK d). Seawater for collection of eDNA and amplicon sequencing of 16S rRNA gene, total of 1L per sample, was pre-filtered through 20 μm pore-size mesh, and then filtered onto 0.2 μm-pore-size polycarbonate filters under low vacuum (47 mm Ø, Whatman® plc, UK). Both filters containing eDNA for 18S rRNA and 16S rRNA gene analysis were placed into cryo-tubes containing 1 mL of sucrose-lysis buffer (50 mM TRIS-HCL, pH 8; 40 mM EDTA, pH 8; 0.75 M sucrose), and then immediately frozen in liquid nitrogen.

Samples for flow cytometry estimation of picoplankton abundances were taken in duplicates of 3 and 5 mL, fixed with 0.1% glutaraldehyde (final conc.), deep frozen in liquid nitrogen and stored at -80°C until further analysis.

Seawater for pigment analysis (total of 1L per sample) was pre-filtered through 20 μm pore-size mesh, then through 3 μm-pore-size polycarbonate filters (47 mm Ø, Whatman® plc, UK) to select for the pico- fraction and finally filtered onto 0.7 µm-pore-size glass microfiber filters - GF/F (47 mm Ø, Whatman® plc, UK). Filters were folded and placed in cryo-tubes and immediately frozen in liquid nitrogen.

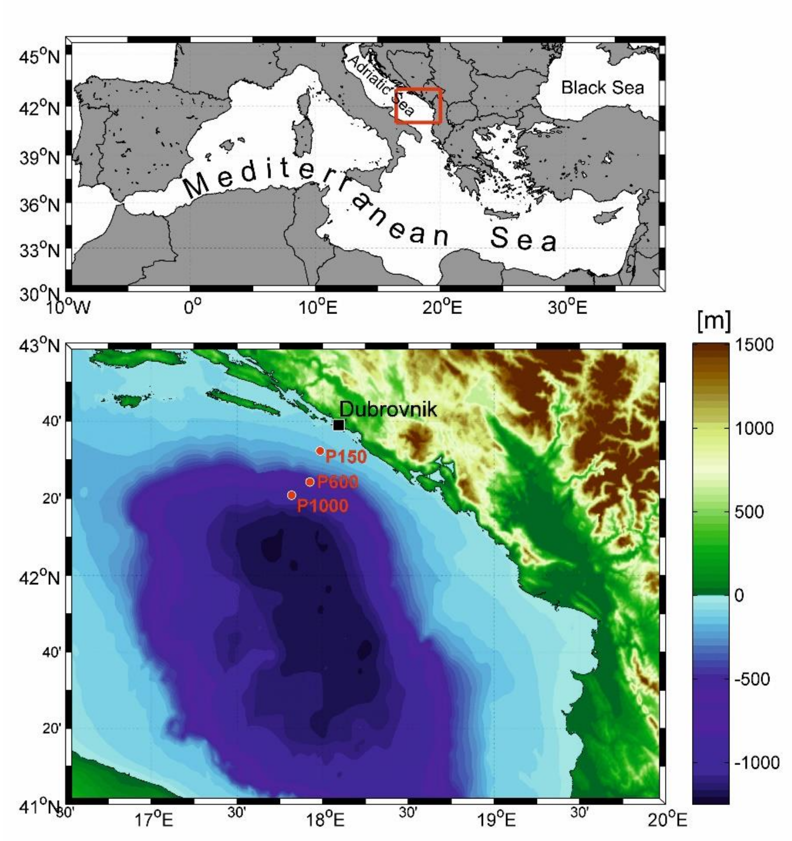

Figure 1. Map of the study area with stations P150, P600 and P1000.

DNA extraction, library preparation and sequencing

Total DNA was extracted from 0.2 µm-pore-size filters (n = 16) as described in Massana et al. (1997). Quality of the extracted DNA was assessed with gel electrophoresis (1 % agarose) and NanoDrop spectrophotometer (BioSpec-nano, Shimadzu, Kyoto, Japan). DNA samples were sent for 16S rRNA and 18S rRNA gene library preparation and Illumina MiSeq amplicon sequencing to Molecular Research MrDNA® (www.mrdnalab.com, Shallowater, TX, USA) using inhouse 27Fmod (5′- AGRGTTTGATCMTGGCTCAG -3′) as forward and 519Rmod (5′- GTNTTACNGCGGCKGCTG -3′) as a reverse primer for Bacteria and Archaea and Reuk454FWD1 (5′- CCAGCASCYGCGGTAATTCC -3′) and ReukREV3 modified (5′- ACTTTCGTTCTTGATYRATGA -3′) for PEs.

The PCR program included a 28 cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) under the following conditions:

94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in 2 % agarose gel to determine the success of amplification and the relative intensity of bands. Sequencing was performed on a MiSeq platform following the manufacturer's guidelines (MrDNA®; www.mrdnalab.com, Shallowater, TX, USA).

16S rRNA gene sequences processing

Obtained raw joined reads were quality checked with FastQC ver. 0.11.5. (Andrews 2010). The 16S rRNA and gene sequences were joined, depleted of barcodes and checked for chimeras by MrDNA® (www.mrdnalab.com, Shallowater, TX, USA). Sequences with read length of more than 250 bp, q value of more than 20 with no ambiguity and homopolymer length of less than 6 bp were selected using split_libraries.py command. From a total of 671,304 raw sequences, in further analyses were included 567,980 sequences (length = min. 250 bp, max. 569 bp and avg. 506 bp) ranging between 62,896 to 76,834 sequences per sample (Table 1). After demultiplexing, sequences were again checked for chimeras and sorted in OTUs by usearch and uchiime (basic usage), generating a total of 1,588 OTUs (Edgar et al. 2011; Edgar 2010). Afterwise, representative sequences were picked with pick_rep_set.py with -m parameter as most_abundant. Taxonomic assignments were carried out using GreenGenes QIIME 1.9.1 compatible database under 97% of identity using assign_taxonomy.py command. Finally, OTU table was produced with make_otu_table.py command. Slight discrepancies for relative abundances between samples processed in this study and the same samples processed by Babić et al. (2018) derive from usage of different database in assignation of taxonomy (GreenGenes vs. SILVA) (Babić et al. 2018). Raw sequences are deposited in the European Nucleotide Archive (ENA) under project number PRJEB23274.

18S rRNA gene sequences processing

Obtained raw joined reads were quality checked with FastQC ver. 0.11.5. (Andrews 2010). The 18S rRNA and gene sequences were joined, depleted of barcodes and checked for chimeras by MrDNA®. Sequences with read length of more than 250 bp, q value of more than 20 with no ambiguity and homopolymer length of less than 6 bp were selected using the split_libraries.py command. From a total of 779,552 raw sequences, 737,293 sequences were included in further analyses (length = min. 252 bp, max. 542 bp and avg. 394 bp) ranging between 71,798 to 129,774 sequences per sample (Table 1). After demultiplexing, sequences were sorted in OTUs by pick_open_reference_otus.py with –r parameter passed using SILVA 123 compatible data file for 18S rRNA gene (97_otus_18S.fasta), generating a total of 1,780 OTUs. Successively, representative sequences were picked with pick_rep_set.py with –m parameter as most_abundant. Taxonomic assignments were carried out using pr2 database using latest pr2 version available 4.7.2 (Guillou et al. 2012) with assign_taxonomy.py. Finally, an OTU table was produced with make_otu_table.py command. Raw sequences are deposited in the European Nucleotide Archive (ENA) under project number PRJEB24945.

Flow cytometry

Abundances of heterotrophic bacteria and cyanobacteria were determined using a BD FACSVerse flow cytometer (BD Biosciences, Franklin Lake, USA) equipped with 488 nm laser excitation and standard filter setup. Cyanobacteria (*Prochlorococcus* and *Synechococcus*) and PPEs cell counts were determined from unstained samples, based on natural fluorescence from phycoerythrin (orange) and chlorophyll (red), as described in Casotti et al. (2003). Heterotrophic bacteria were enumerated as in Balestra et al. (2011) after staining with SYBRGreen I (Lonza, final concentration 10^{-3} of stock solution) for 15 min in the dark at room temperature prior to analysis. The threshold was set to green fluorescence. Heterotrophic prokaryotes were identified in plots of red fluorescence versus green fluorescence and side scatter versus green fluorescence. All data were acquired in log mode. 1 μm fluorescent polystyrene calibration beads (Fluoresbrite YG Microsphere, Polysciences Inc. Warrington, PA, USA) were added to all samples as internal standard. Data acquisition was performed using FACSuite software (BD Biosciences, Franklin Lake, USA) and the files analysed using FCS Express 4 Plus Flow Research Edition software (DeNovo Software, Glendale, USA).

Pigment analysis

GF/F filters were defrosted and pigment extracted in 4 mL of cold 90% acetone by sonication, and the extracts clarified by centrifugation. The pigments were separated by a reversed phase High-performance Liquid Cromatography (HPLC) as in Šilović et al. (2011).

Data analysis

Alpha diversity indices such as Chao1, observed OTUs, Simpson and Shannon, were calculated using alpha_diversity.py command in QIIME 1.9.1 (Caporaso et al. 2010). To visualize taxonomical composition, bar-plots and pie-charts indicating percentage of detected OTUs and cluster dendrograms with sample separation based on calculated Bray-Curtis dissimilarity distance matrix, were generated using R software (version 3.4.3) (R Development Core Team, 2008) using the ″vegan″ and ″ggplot2″ packages (Oksanen et al. 2007, Wickham 2009). Detailed taxonomic assignments were visualised and examined in MEGAN version 6.10.8. (community edition) (Huson et al. 2016).

RESULTS

Picoeukaryote diversity: taxonomical composition and species richness

In total 737,293 good quality sequences were obtained from 8 different seawater samples, ranging from 71,798 to 129,774 reads per sample (Table 1). Clustering of these reads to PR2 database resulted in 1,780 OTUs (ranging from 888 to 1,085 OTUs per sample), 1,210 of which belonged to Dinophyta. Next most represented taxon was Stramenopiles with 194 OTUs, Radiolaria with 148 OTUs, Hacrobia with 65 OTUs and Archaeplastida with 41 OTUs. The remaining 122 OTUs were attributed to Ciliophora, Alveolata, Amoebozoa, Choanoflagellatea (Opisthokonta) and Fungi. 55 OTUs (972 sequences) were identified as Metazoa, probably from larvae, commonly present as contaminant (Romari & Vaulot 2004; Shi et al. 2009).

Picoeukaryotes with relative abundances higher than 1% of total sequences were represented by Dinophyta (88.37%), Rhizaria (6.25%), Hacrobia (1.88%), Archaeplastida (1.61%) and Stramenopiles (1.33%) (Figure 2B). Dinophyta were dominant in all the samples, with the lowest relative abundance recorded at P1000-80m (70.62%) where higher relative abundance of Rhizaria occurred (23.19%). Similarly, at P1000-200m, Dinophyta (80.14%) relative abundance decreased while Rhizaria increased (18.35%) (Figure 2A). PPEs were represented by Archaeplastida, photosynthetic representatives of the Stramenopiles and small amount of Haptophyta, together reaching maximum relative abundance at P600-150m of 5% of the photoautotrophs (Figure 2A).

Further analysis of PEs taxonomic composition at lower taxonomical levels revealed that dominant dinoflagellates belonged to the parasitic order of Syndiniales (79.67%) (Figure 3B) and ranged from 74.96% at P600-25m to 89.11% at P150-30m (Figure 3A). Overall, Syndiniales Dino-Group-II had higher relative abundance than Syndiniales Dino-Group-I, 40.96% and 38.71%, respectively (Figure 3B). Within Syndiniales Dino-Group-I, clade 4

(17.3 %), followed by clade 1 and 5 (15.2% and 4.9%) were dominant. Within Syndiniales Dino-Group-II, clades 10 and 11 (12.2%), followed by clades 7 and 6 (7.2% and 5.1%) were dominant. Radiolarians were mostly present at P1000-80m and P1000-200m (Figures 2A and 3A). In sample P1000-80m, almost all Rhizaria sequences were identified as Radiolaria, Collodaria, family Sphaerozoidae (22.1%), while in sample P1000-200m rhizarian sequences were identified as Radiolaria, order Spumellaria (5.33%), Acantharea (3.22%), Nessellaria (0.86%), and other radiolarians (8.94%). PPEs (Archaeplastida and Stramenopiles) were represented by Mamiellophyceae (Bathycoccaceae and Mamiellaceae, 1.6%), Cryptophyceae (Cryptomonadales, 1.1%), other Chlorophytes (Nephroselmidales, Prasinophyceae Clade VIIA and 9B, 0.01%), Prymnesiophyceae, Bacillariophyta (Mediophyceae, Coscinodiscophyceae and Bacillariophyceae), Bolidophyceae, Crysophyceae, Dictyochophyceae and Pelagophyceae whose individual relative abundance never exceed 1%.

Alpha diversity indices (Chao1, Shannon and Simpson; Table 1) were calculated after random sub-sampling of all samples at even depth (70,720 sequences, starting from 7,081 sequence, iterating every 7,071 sequence). Average Chao1 index for 18S rRNA gene was 1328.38, while average Shannon and Simpson indices were 6.36 and 0.95, respectively (Table 1). Considering all indices, general biodiversity based on 18S rRNA gene was high (Table 1), with the lowest diversity recorded at P600-75m, and the highest at P150-100m (Table 1).

Table 1. Values of the Alpha-diversity indices calculated from 11 iterations for 70,720 min. sequences number per sample for 18S rRNA gene and from 11 iterations for 47,870 min. sequences number per sample for 16S rRNA gene.

	Sample	Good quality sequences	Observed OTUs	Chao1	Shannon	Simpson
18S rRNA gene	P150-30m	81,712	1053	1355.79	6.62	0.97
	P150-100m	82,376	1039	1324.60	6.79	0.98
	P600-25m	85,645	1078	1438.46	6.82	0.97
	P600-75m	71,798	888	1133.32	5.53	0.89
	P600-150m	86,890	1054	1429.58	6.37	0.95
	P1000-80m	129,774	1112	1409.90	5.98	0.93
	P1000-100m	113,550	1085	1346.37	6.50	0.96

	P1000-200m	85,548	962	1189.05	6.26	0.95
	Average	***92,162***	***1034***	***1328.38***	***6.36***	***0.95***
16S rRNA gene	P150-30m	73,282	660	884.30	6.50	0.97
	P150-100m	76,834	709	902.29	6.71	0.98
	P600-25m	69,663	668	873.74	5.97	0.94
	P600-75m	75,980	703	892.01	6.45	0.97
	P600-150m	66,297	681	904.56	6.72	0.98
	P1000-80m	69,661	647	837.31	6.56	0.97
	P1000-100m	79,678	717	1005.44	6.63	0.97
	P1000-200m	68,657	715	905.94	6.94	0.98
	Average	***72,506***	***687***	***901***	***6.56***	***0.97***

Bacterial diversity: taxonomic composition and species richness indices

A total of 567,920 good quality sequences were obtained ranging between 66,297 to 79,678 reads per sample (Table 1). Clustering of these reads to GreenGenes database with a 97% threshold similarity resulted in 1,588 OTUs (647 to 717 OTUs per sample), of which 498 belonged to *Alphaproteobacteria*. Next most represented taxon was *Gammaproteobacteria* with 352 OTUs, *Bacteroidetes* with 136 OTUs, *Cyanobacteria* with 121 OTUs, *Marinimicrobia* (SAR406) with 84 OTUs, *Firmicutes* with 74 OTUs, *Deltaproteobacteria* with 73 OTUs and *Chloroflexi* with 50 OTUs. The remaining 200 OTUs were represented by *Actinobacteria*, *Acidobacteria*, *Betaproteobacteria*, *Planctomycetes*, *Verrucomicrobia* and other Bacteria. No Archaea sequences were identified in the 8 samples.

Bacteria with relative abundance higher than 1% of total sequences were represented by *Alphaproteobacteria* (44.91%), *Gammaproteobacteria* (14.96%), *Bacteroidetes* (13%), *Cyanobacteria* (9.52%), *Marinimicrobia* (SAR406, 7.97%), *Deltaproteobacteria* (3.83%), *Actinobacteria* (2.24%) and *Chloroflexi* (1.90%) (Figure 2D). *Alphaproteobacteria* were dominant in all the samples, while the highest relative abundance of *Gammaproteobacteria* and *Cyanobacteria*, was 27.13% and 24.41%, respectively (Figure 2C). A significant increase in relative abundance of SAR406 and *Chloroflexi* was recorded at P1000-80m (18.37% and

6.45%, respectively), where the lowest relative abundance of *Bacteroidetes* occurred as well (1.45%, Figure 2C). Interestingly, just 20m below, at P1000-100m, the same showed its highest relative abundance (24.36%, Figure 2C). The phototrophic Cyanobacteria showed relative abundances between 0.47% (P1000-80m) and 25.41% (P600-25m) (Figure 2C).

Further analysis of bacterial taxonomic composition at lower taxonomical level revealed that *Pelagibacteraceae* were the dominant *Alphaproteobacteria* together with SAR11 clade (40.78%) (Figure 3D). SAR11 was dominant in all samples, ranging from 30.90% at P600-25m to 50.21% at P1000-200m (Figure 3C). *Gammaproteobacteria* were represented by *Oceanospirillales* and *Pseudomonadales*, whose relative abundances complemented each other: when *Oceanospirillales* dominated *Gammaroteobacteria*, *Pseudomonadales* were scarce and the other way around (Figure 3C). *Bacteroidetes* were mainly composed of *Flavobacteriales* (10.18%, Figure 3D) with the highest relative abundance at P600-150m (13.44%), and the lowest at P600-25m (5.66%) (Figure 3C). Among Cyanobacteria, *Prochlorococcus* and *Synechococcus* were most abundant, with a general dominance of *Synechococcus* over *Prochlorococcus* (5.15% over 1.17%, respectively) (Figure 3D). More detailed analysis in *Deltaproteobacteria* revealed dominance of SAR324 clade (3.67%, Figure 3D), with highest abundances reported at P1000-80m (16.05%) and lowest at P600-25m (0.38%) (Figure 3C). The remaining taxonomical level identification did not reveal prevalence of any family of genera in particular (Figure 3C).

Alpha diversity indices (Chao1, Shannon and Simpson; Table 1) were calculated after random sub-sampling of all samples at even depth (47,870 sequences, starting from 10 iterating every 4,786 sequence). Average Chao1 index for 16S rRNA gene was 901, while average Shannon and Simpson indices were 6.56 and 0.97, respectively (Table 1). Considering all indices, general biodiversity based on 16S rRNA gene was high (Table 1). The lowest diversity, considering all indices, was recorded at P600-25m, while the highest was recorded at P150-100m (Table 1).

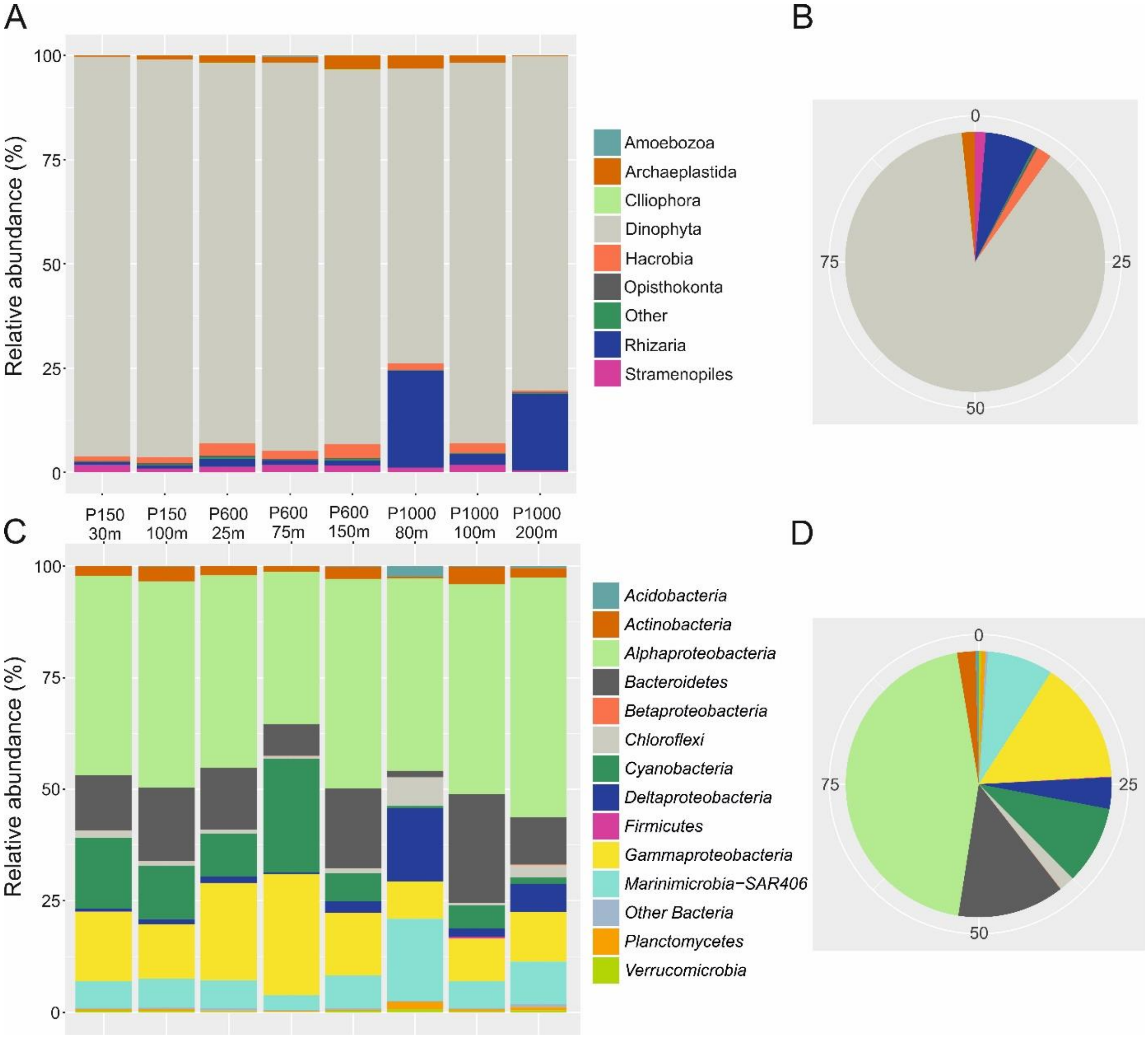

Figure 2. Relative abundance of the bacterial, cyanobacterial and picoeukaryotic taxa at each station and in total. A: Relative abundance of the picoeukaryotic taxa represented as higher taxonomic groups. B: Taxonomic distribution of all picoeukaryotic sequences retrieved in this study, represented as higher taxonomic groups C: Relative abundance of the bacterial taxa represented at the class and phylum level. D: Taxonomic distribution of all bacterial sequences retrieved in this study, represented at the class and phylum level.

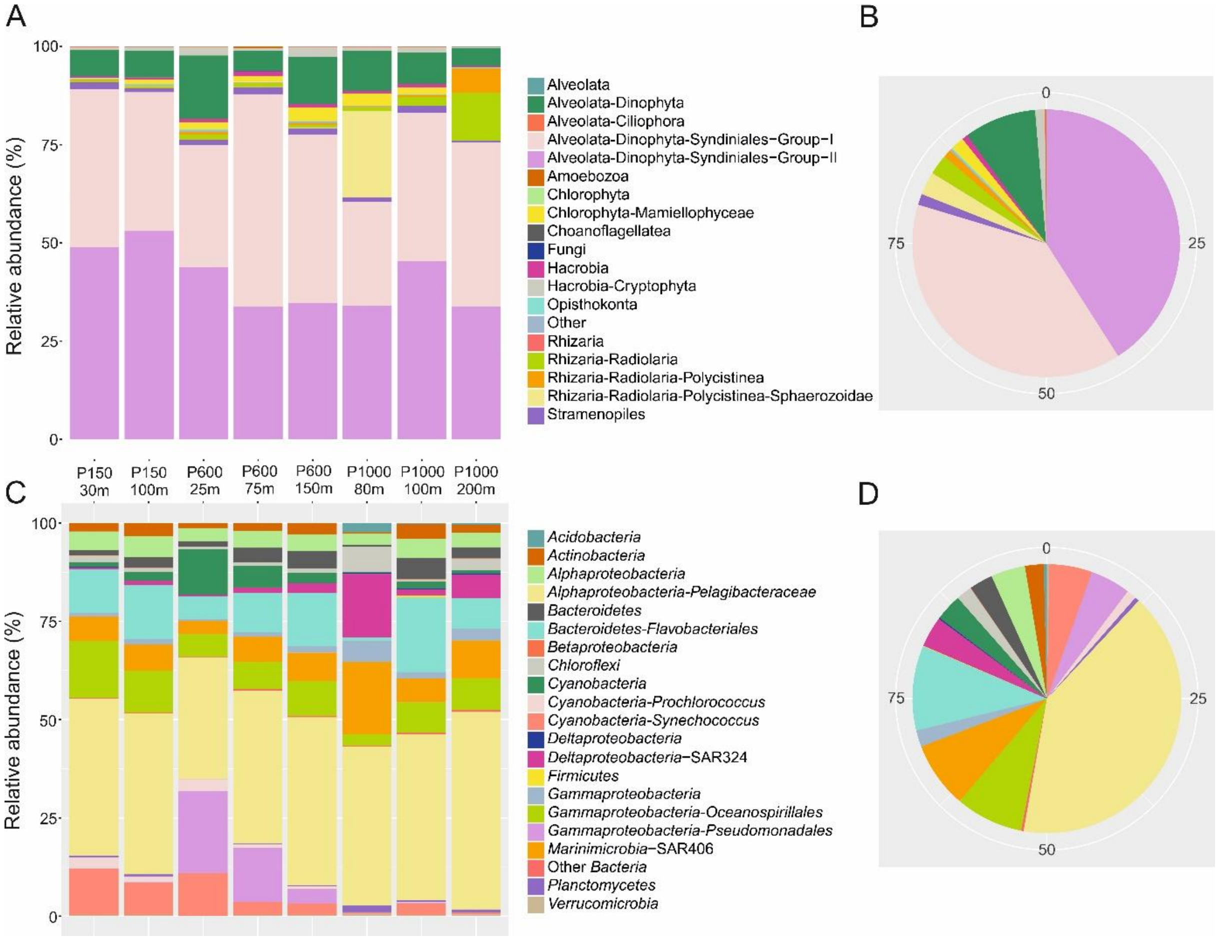

Figure 3. Relative abundance of the bacterial, cyanobacterial and picoeukaryotic taxa at each station and in total. A: Relative abundance of the picoeukaryotic taxa represented at higer taxonomic level. B: Taxonomic distribution of all picoeukaryotic sequences retrieved in this study, represented at higher taxonomic level. C: Relative abundance of the bacterial taxa represented at lower taxonomic level. D: Taxonomic distribution of all bacterial sequences retrieved in this study, represented at the lowest taxonomic level.

Beta diversity of bacterial and PEs communities

Beta-diversity analysis of 18S rRNA and 16S rRNA gene sequences revealed a separation of samples into three groups, somewhat different regarding each gene (Figures 3 A and B). Bray-Curtis distance between the three groups for 18S rRNA gene ranged from 0.21 to 0.55 (Figure 3A), while for 16S rRNA gene ranged from 0.14 to 0.83 (Figure 3B). The most relevant difference in grouping comparing 18S rRNA and 16S rRNA gene sequences occurred in sample P1000-80m, which in 16S rRNA dataset forms separated group, indicating an unique bacterial community, while for 18S rRNA dataset the most different samples were P600-75m and P1000-200m (Figures 3 A and B).

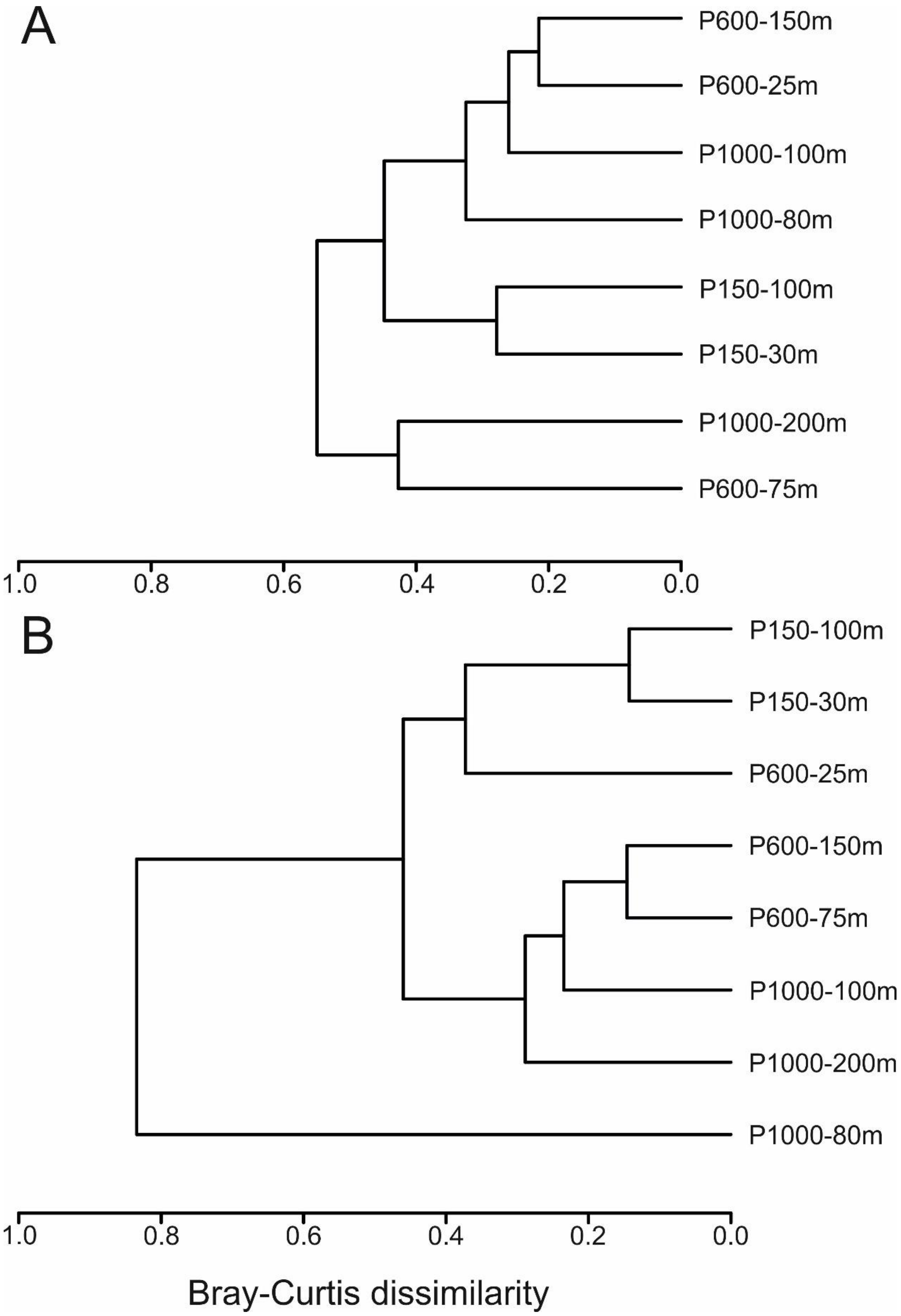

Figure 4. A: Bray-Curtis bacterial dissimilarity dendrogram derived from distance matrix. B: Bray-Curtis picoeukaryote dissimilarity dendrogram derived from distance matrix.

Flow cytometry and photosynthetic pigment composition

The highest abundances of *Synechococcus*, *Prochlorococcus* and PPEs were recorded at P600-25m (2.5×10^4 and 2.3×10^4 and 3×10^3 cells mL^{-1}, respectively), where also the highest Chl *a* concentration was measured (12.12 µg $L^{-}1$; Table 2). At the same station, 50 m below, the heterotrophic bacteria had their maximum (7×10^5 cells mL^{-1}; Table 2).

8 different pigments were detected (Table 2), marking different taxonomical linages within picophytoplankton. Cyanobacterial genera *Prochlorococcus* and *Synechococcus* were detected by the presence of divinyl chl *a* and zeaxanthin at P150-30m, P600-25m and P600-75m. The presence of PPEs classes was supported by the presence of chlorophyll *c3* and 19'-butanoiloxyfucoxanthin *(*Prymnesiophyceae, Chrysophyceae), 19'-hexanoiloxyfucoxanthin (Prymnesiophyceae), fucoxanthin (Bacillariophyceae, Prymnesiophyceae, Chrysophyceae) and prasinoxanthin (Prasinophyceae). All pigments (except 19'-hexanoiloxyfucoxanthin, zeaxanthin and divinyl chl *a*) had their maximum values at P600-25m (Table 2).

Table 2. Flow-cytometrically determined concentrations of picoplankton (cells mL^{-1}) and HPLC-determined photosynthetic pigments concentrations (µg L^{-1}). *Syn* – *Synechococcus*; *Pro- Prochlorococcus*; PPEs – photosynthetic picoeukaryotes; HB- heterotrophic bacteria; Chl *c3* – chlorophyll *c3*; But- 19'-Butanoiloxyfucoxanthin; Fuco- Fucoxanthin; Hex- 19'-Hexanoiloxyfucoxanthin; Prasino- Prasinoxanthin; Zea- Zeaxanthin; Dvchl *a*- divinyl chlorophyll *a*; Chl *a*- chlorophyll *a*; **n/d**- not detected

Sample	***Syn***	***Pro***	**PPEs**	**HB**	**Chl *c3***	**But**	**Fuco**	**Hex**	**Prasino**	**Zea**	**Dvchl *a***	**Chl *a***
P150-30m	18700	14660	1030	557130	8.00	3.14	**n/d**	3.93	**n/d**	2.40	1.20	10.33
P150-100m	8030	7860	1880	465690	2.72	1.85	**n/d**	7.40	**n/d**	**n/d**	**n/d**	4.60
P600-25m	25150	23460	3050	755080	7.05	3.10	0.40	4.72	1.60	2.20	0.92	12.12
P600-75m	5370	7480	2760	782600	4.54	3.00	0.31	5.90	**n/d**	0.80	0.50	5.30
P600-150m	3040	4000	1370	699110	1.90	0.83	0.17	1.00	**n/d**	**n/d**	**n/d**	1.80
P1000-80m	5680	6870	1920	690800	3.20	1.22	**n/d**	1.10	**n/d**	**n/d**	**n/d**	3.50
P1000-100m	2270	2640	1150	674900	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**	1.03
P1000-200m	460	150	100	541600	2.04	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**	1.13

Discussion

This study confirms small coccoid cyanobacteria *Prochlorococcus* and *Synechococcus* as dominat picoplankton primary producers in the extreme oligotrophic South Adriatic Sea. *Synechococcus* is a ubiquitous genus in the marine environment, represented by many serogroups, although it is more abundant in nutrient-rich regions, whereas *Prochlorococcus* is more restricted to oligotrophic tropical and sub-tropical waters (Partensky, Hess & Vaulot, 1999; Bouman et al. 2011). In the southern Adriatic Sea, *Prochlorococcus* and *Synechococcus* are both present in high numbers, except when deep convection mixing events boost up PPEs and down *Synechococcus* abundances, completely eliminating *Prochlorococcus* (Šilović et al. 2018). Šilović et al. (2018) also showed that *Prochlorococcus* abundances greatly increase with increasing salinity brought by the LIW, injecting Mediterranean high-salinity deep water into the southern Adriatic Sea. Nevertheless, molecular techniques showed dominance of *Synechococcus* over *Prochlorococcus* in the South Adriatic Sea during both convection mixing events and semi-stratified events (Korlević et al. 2015; Babić et al. 2018). Despite the relatively high numbers of PPEs estimated by flow cytometry, HTS showed extremely low number of sequences attributable to PPEs, with HPEs counterparts dominating the community. This finding is similar to what observed in other oligotrophic environments, such as the Red Sea, the Atlantic and Pacific Oceans both in sunlit parts and deep parts of the water column (Shi et al. 2009, Acosta et al. 2013, De Vargas et al. 2015, Pernice et al. 2015, Pearman et al. 2017).

The general dominance of picoplankton in oligotrophic areas has been observed worldwide (Caron et al, 1999, Ignatiades et al. 2002, Siokou-Frangou et al. 2010). Some authors have shown that increasing gradients of turbulence (mixing of the water column by external forces such as tides, upwelling or winds) and nutrient concentrations are the main drivers in shaping plankton community structure (Margalef 1978, Cullen et al. 2002). Low nutrient and low turbulence areas are dominated by picophytoplankton, escpecially dinoflagellates and slow growing groups with specialist strategies (e.g. mixotrophy) (Cullen et al. 2002, Gilbert 2016). In case of this study, however, the dominance of heterotrophs in picoplankton (heterotrophic bacteria and HPEs) is consistent with AOU measurements reported by Babić et al. (2018) who found that the main process in investigated area was respiration, not primary production. Therefore, heterotrophic bacteria and then HPEs are suggested as main drivers of the southern Adriatic Sea ecosystem.

The main dominant picoeukaryotic group in our samples belonged to Alveolata, classes Dinophyceae and Syndiniophyceae (groups I and II with clades 1 and 5 and 10+11, 7 and 6, respectively). Syndiniales are a parasitic order of dinoflagellates who belong to recently discovered MALV Group II, which have been retrieved from various marine habitats, mainly from the picoplankton fraction (<2 or <3 µm size fractionated samples) (Díez et al., 2001, Moon-van der Staay et al., 2001, Guillou et al. 2008, Massana & Pedrós-Alió, 2008). This is congruent with studies of Pearman et al. (2017) and Acosta et al. (2013) from the Red Sea and other oligotrophic regions (De Vargas et al. 2015; Estrada et al. 2016). As most of dinoflagellates are mixotrophs, they gain energy from sunlight and at the same time acquire inorganic nutrient requirements and essential organic nutrients, such as amino acids and vitamins *via* bacterivory (Unrein et al. 2007; Hartmann et al., 2012). Additionally, mixotrophy favours dinoflagellate propagation in oligotrophic conditions, where grazing is reported to be higher (Wilken et al. 2013). However, there is also a possibility that these data are overestimated, considering that dinoflagellates can have up to 12,000 copies of 18S rRNA gene (Zhu et al. 2005; Not et al. 2009).

Dominance of a reduced number of OTUs, as found in this study, is not unusual. Keeling & del Campo (2017) analysed the *Tara* Ocean dataset of 18S rRNA V9 tag sequences and found that 8 OTUs represented more than 50% of the reads, belonging to radiolarians and dinoflagellates. These authors identified some OTUs as ‘jackpots’, indicating lineages that are dominated by a single OTU and some OTUs as ‘normal’, indicating lineages where significant proportion of the reads from the entire group are distributed across less abundant OTUs. As an exception, Keeling & del Campo (2017) emphasize Syndinians who were an extreme case where distribution across the ten most abundant OTUs was nearly equal, suggesting that relative abundance of sequences in each OTU was similar to the remaining ones, which is the case in this study as well.

The second picoeukaryotic group in this study belonged to radiolarians, orders Collodaria (Sphaerozoidae, *Collozoum inerme*), Spumellaria, Acantharea, Nessellaria, and others. Radiolarians are skeleton-bearing marine heterotrophic protists belonging to the eukaryotic phylum Retaria, which is included within the super-group Rhizaria (Nikolaev et al., 2004; Adl et al., 2005; Moreira et al., 2007). Six well established orders divide based on the structure of their skeletons: Acantharia possess a skeleton made of strontium sulfate, while Entactinaria, Taxopodia, Collodaria, Nessellaria and Spumellaria have a skeleton made of opaline silica (Suzuki and Not, 2015). Each collodarian colony is composed of hundreds of thousands of collodarian cells embedded in a gelatinous matrix, while their reproductive cells are flagellated and between 2 and 10µm in size, so called “swarmers” (Anderson, 1983), which can explain their large relative abundance in the pico-fraction in this study. Additionally, Collodaria have 5770±1960 18S rDNA gene copies per cell, that in colonies can add up to 37474±17799 gene copies (Biard et al. 2017, and this can explain their large relative abundances in our sample P1000-80m). Amplicon-based analyses are influenced by a number of factors that bias quantitative interpretation with respect to cell abundance. These include variations in gene copy number, intra-genomic rRNA gene polymorphisms (Pillet et al., 2012), differential polymerase chain reaction (PCR) recovery (due to amplicon size and primer bias), and/or sequencing artifacts (Kebschull and Zador, 2015). Collodaria are still unknown with respect to their feeding behaviour and occurrence of photosymbiosis, as reported in the dinoflagellate *Brandtodinium nutricula* (Brandt) Probert & Siano (Hollande & Enjumet, 1953; Probert et al. 2014). In this study, most of the sequences attributed to Collodaria were from *Collozoum inerme* (J. Müller, 1856), a species commonly known to have photoautotrophic dinoflagellates as symbionts (″zooxanthella″, belonging to the genus *Brandtodinium*, Probert et al. 2014).

Different radiolarians (Spumellaria, Acantharea, Nessellaria and other unidentified radiolarians) were, instead, observed at P1000-200m, suggesting that different environmental conditions occurred at this depth when compared to the other samples. Spumellaria and Nessellaria are known to hold photosymbiosis, in most cases with only one dinoflagellate species, *B. nutricula* (Stal & Cretoiu 2016). This could indicate increased photosynthetic activity at P1000-200m, where chl *a* was still detected with a concentration similar to that at P1000-100m, where, in turn, no colonial radiolarians and very little phototrophs were present. Interestingly, not even a single sequence belonging to other families of Collodaria – Collosphaeridae was found, although Biard et al. (2017) had detected both Collosphaeridae and Sphaerozoidae in equal ratio during the *Tara* Oceans survey.

Generally, low relative abundances of PPEs among sequences is a common feature of oligotrophic environments, as also found by other authors (De Vargas et al. 2015, Monier et al. 2016). Most of our PPEs sequences belonged to Mamiellophyceae, which is unusual in temperate areas, where Trebouxiophyceae prevail, instead (Tragin et al. 2017). On the other hand, our data do not allow to fully assess dominance of Mamiellophyceae or Trebouxiophyceae or any other photosynthetic taxa group, as the whole photoautotrophic community did not exceed 5% of total community, as revealed by HTS. Members of prasinophytes (Mamiellophyceae) proved to be abundant (or even dominant) in various regions of the world's oceans enriched with nutrients, coastal regions (Guillou et al. 2004; Romari and Vaulot 2004; Worden 2006), while groups of uncultured prymnesiophytes, chrysophytes, and pelagophytes are often more dominant in open ocean waters (Fuller et al. 2006; Shi et al. 2009; Cuvelier et al. 2010). The low proportion of photoautotrophs in our pico-fraction can be explained by the fact that many of these organisms can live either photoautotrophically or mixotrophically (as phagotrophs), and in many cases it is quite hard, if not impossible to truly characterize their ecological role in the environment (Zubkov and Tarran, 2008; Caron et al., 2009; Hartmann et al., 2012).

The bacterial community showed a structure typical of usuallyoligotrophic environment, as already reported by other authors in the same area (Korlević et al. 2015; Babić et al. 2018). Other studies from the Mediterranean Sea and the Atlantic Ocean also showed *Alphaproteobacteria* and *Gammaproteobacteria* as dominant, representing more than 50% of the total bacteria (Alonso-Sáez et al. 2007, Feingersch et al. 2009, Zinger et al. 2011). Among *Alphaproteobacteria*, family *Pelagibacteraceae* with SAR11 clade dominated as K-strategist, once again confirming that this lifestyle is predominant in oligotrophic conditions (Babić et al. 2018). *Gammaproteobacteria* were also often closely following *Alphaproteobacteria* in the southern Adriatic Sea, except after deep convection event and following full vertical mixing of the water column, when they virtually disappeared (<3%, Korlević et al. 2015). In our study *Gammaproteobacteria* were co-dominant with *Alphaproteobacteria,* indicating limited mixing during 2016, which is confirmed by Babić et al. (2018) who clearly divided photic from aphotic bacterial communities in correlation to depth of a semi-stratified water column. *Bacteroidetes*, who are usually negatively correlated with depth, represented the third prevailing bacterial class in relative abundance, as also found by Korlević et al. (2015) and Babić et al. (2018). *Cyanobacteria*, our fourth most represented group, showed a clear dominance of *Synechococcus* over *Prochlorococcus,* which is consistent with our studies in the southern Adriatic Sea surface waters (Korlević et al. 2015; Babić et al. 2018) and for surface oceans in general (Schattenhofer et al. 2009; Brown et al. 2009). Presence, but not dominance, of *Marinimicrobia* SAR406 clade in our samples is also consistent with its reported occurrence in the euphotic layer in general (Yilmaz et al. 2016). Relatively lower abundances of *Deltaproteobacteria* (SAR324 clade), *Actinobacteria* and *Chloroflexi* (SAR202 clade) confirm vertical distributions of these bacteria in world oceans, being primarily very deep-ocean clades (Yilmaz et al. 2016; Quaiser et al. 2011; Morris et al. 2004).

Prochlorococcus, *Synechococcus*, PPEs and heterotrophic bacteria abundances were consistent with previous findings (Najdek et al. 2014; Korlević et al. 2015). Viličić et al. (2010) investigated phytoplankton by microscopy and flow cytometry in the south-eastern Adriatic Sea in spring and recorded higher PPEs numbers (3.8 – 4.5×10^3 cells mL^{-1}), accumulating at offshore stations at 50 m depth, while cyanobacteria (9×10^4 cells mL^{-1}) accumulated at station closer to the coast also at 50 m depth. In the same study, bacteria did not show any accumulation pattern, but were high in numbers throughout the water column at coastal as well as at offshore stations (Viličić et al. 2010). Also, Šilović et al. (2011) investigated the same area during spring of 2011 and recorded similar abundances. In our study, cyanobacteria and PPEs did not show accumulation at specific depths, but were equally dispersed throughout euphotic zone, decreasing with depth, which can be explained by a well-mixed upper water column. P1000-80m proved to be an exception to this pattern, having different bacterial and PE communities, suggesting an intrusion of water with different physico-chemical characteristics, allowing different organisms to flourish. At this site Babić et al. (2018) reported lower concentrations of chl *a*, lower dissolved oxygen concentrations, increased POC and bacterial community composition specific to aphotic water layers, and here we can additionally support their conclusions based on the occurrence of high collodarian sequence numbers.

Synechococcus and *Prochlorococcus* counts appear to be in the range of previous research in oligotrophic environments, with *Synechococcus* being dominant in coastal and *Prochloroccus* in offshore waters (Partensky et al. 1999; van den Engh et al. 2017). The highest recorded numbers of *Synechococcus* and *Prochlorococcus* in this study correlated with higher concentrations of zeaxanthin plus β-carotene (for *Synechococcus*) and divinyl chlorophyll *a* (for *Prochlorococcus*), which are considered as taxonomical markers of these cyanobacteria (Waterbury et al. 1979; Chisholm et al. 1992; Roy et al. 2011). These pigments were detected in just three samples (P150-30m, P600-25m and P600-75m) although *Synechococcus* and *Prochlorochoccus* were detected by flow cytometry in all samples. This may indicate a threshold of 5000 cells mL^{-1} for *Synechococcus* and 7000 cells mL^{-1} for *Prochloroccus* for the HPLC method. Although, at P150-100m counts were higher than this threshold but no pigment was detected. Apart from errors during the extraction or from machine limitations (Claustre et al. 2004), it is also possible that the content of pigment per cell was different, as related to a different light history or the water column stability.

The pigment assemblage at P150 station (accounting for chlorophyll *c3*, 19’-butanoiloxyfucoxanthin, 19’- hexanoiloxifucoxanthin, divinyl chl *a*, zeaxanthin and chl *a*) indicated the presence of cyanobacteria (*Synechococcus* and *Prochlorococcus*), prymnesiophytes, diatoms and haptophytes (Roy et al. 2011), also confirmed by flow cytometry counts as well as HTS. Higher concentrations of fucoxanthin, 19’-butanoiloxyfucoxanthin and prasinoxanthin were detected at P600-25m, indicating presence of prasinophytes, prymnesiophytes and chrysophytes. This matched higher PPEs numbers by flow cytometry and HTS results, the latter with Prasino-Clade IX recorded in higher relative abundances at P600-25m and P600-75m than in other samples (0.0013%), and total Chrysophyceae and Primnesiophyceae with their maxima (0.021% and 0.0013%, respectively). In deeper samples (P1000-100m, P600-150m, and P1000-200m) photosynthetic pigments suggested the presence of diatoms which is also supported by higher relative abundances revealed by HTS, diatoms having 0.1958%, 0.1775% and 0.0425%, respectively. Together with flow cytometry, pigment composition of pico-fraction provided us with a better insight of the photoautotrophic picoplankton.

Conclusions

This study provides the first snapshot of the PEs diversity present in oligotrophic euphotic waters of the southern Adriatic Sea, hence setting the stage for large-scale surveying and characterization of the eukaryotic diversity in the entire basin. The photosynthetic component of picoplankton was dominated by cyanobacteria, while the PEs proved to be 95% hetero- or mixo- trophic, with just 5% obligatory photoautotrophs. HPEs dominate both in lineages and in OTU numbers, indicating the need for a better understanding of their role in the ecosystem. The bacterial community structure is confirmed as compared to other oligotrophic areas of the world ocean and the same site in other seasons. The integrated approach used, coupling flow cytometry, pigment analysis and sequencing of marker genes proved to be valuable in complementing information and providing a clearer picture of the community composition to be related to their possible role in the microbial loop.

Acknowledgments

This study was fully supported by the Croatian Science Foundation under the project BIOTA (Bio-tracing Adriatic Water Masses) UIP-2013-11-6433. Authors want to thank the crew of R/V ″Naše More″ for their help during the fieldwork and the Flow Cytometry Facility of the Stazione Zoologica Anton Dohrn for the analyses.

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DISCUSSION

„Discussion is an exchange of knowledge; argument an exchange of ignorance."- Robert Quillen.

The Adriatic Sea - a 'hotspot' of biodiversity

All the aims and questions within this thesis were addressed and answered through seven publications enclosed. Starting with the first aim, publications **I-VI** all directly contribute with the characterizations of cultivated strains of diatom genera *Entomoneis*, *Pseudo-nitzschia* and *Haslea*, as well as PEs genus *Picochlorum*. Those characterizations and descriptions of new species were made according to the newest available literature, using all available methodology (light and electron microscopy, molecular identification using multiple genetic markers and physiological attributes characterization in case of *Picochlorum* sp.) (Henley et al., 2004; Amato and Montresor, 2008; Ruck and Theriot, 2011; Gastineau et al. 2014, 2016; Theriot et al., 2015; Ruck et al. 2016; Li et al., 2017; Pinseel et al., 2016, 2017). The second thesis aim was directly met with the publication **VII**, the first study dealing with picoeukaryotic next generation sequencing dataset derived from the Adriatic Sea. Again, this publication is congruent with the latest studies considering methodology, as well as with their results, indicating high number of heterotrophic organisms in oligotrophic ecosystems (Shi et al., 2009, Acosta et al., 2013, De Vargas et al., 2015, Pernice et al., 2015, Pearman et al., 2017). The third aim was addressed with publications **I** and **II**, in which seven new species of planktonic pennate diatoms from genus *Entomoneis* were described. This discovery is particularly important since genus *Entomoneis* was not considered to be that diverse in marine plankton, as well as these publications were also the first ones to consider using multiple species concepts to define seven different species in genus *Entomoneis*. Additionally, these publications introduced some new terminology in morphology of the genus *Entomoneis*, as well as filled the gap in publicly open databases (GenBank, ENA, Algaebase) with newly deposited sequences and information of organisms which we presume will be detected globally in the future.

Additional questions asked through this thesis were completely or partially answered with given publications. First, and probably the most difficult thesis question *"Where is the phylogenetical border between species and genus in picoeukaryotes and planktonic pennate diatoms? Does the current knowledge of species/genus border adequately reflect the use and availability of new gene markers in picoeukaryotes and planktonic pennate diatom research and microscopy in classical morphology?"* was answered through results in publications **I-IV** and **VI**. In these publications, we used different available genetic markers and light and electron microscopy on live cultivated cells and cells observed in the natural material to delineate species from genus level. Publications **I** and **II** are not just pioneer studies for the genus *Entomoneis* that combine morphology and phylogeny in species identification, but they also represent first species descriptions of planktonic pennate diatoms in the Adriatic Sea. However, here it is important to emphasize that the **IInd** publication would not have been possible if the cultivation of first *Entomoneis* species – *E. tenera* – had not been successful and if cells of the genus *Entomoneis* had been overlooked in the field samples once again. We would probably not focus on cultivation of so many *Entomoneis* strains in the first place if cells were not easy to isolate in monoclonal cultures, or if they had not been abundant in both phytoplankton net samples and fractioned samples, which was aimed at the pico-fraction smaller than 3.0 µm. This is particularly interesting, as all the cells of *Entomoneis* strains are bigger than 3.0 µm, but have managed to 'squeeze' through the small pores due to their light silification and ability to twist around their apical or transapical axis. Many known pennate diatom cells are able to contaminate fractioned samples simply because they are thin, so when turned upright in parallel to their apical axis, they go through the pores without any problem (Vaulot et al., 2008; Belevich et al., 2018). Additionally, we showed the same contamination pattern in amplicon sequenced pico-fraction in publication **VII**, where we detected sequences belonging to both pennate and centric diatoms, indicating cell breakage or passing through filter pores. Likewise, these cells were not that abundant in field material (small *Entomoneis* cells appeared in approx. 10% of the samples), but they were all present in cultured material as 'weed-species'. Reasons for this can be numerous, but the most obvious one is removal of predators (in this case by filtration, than by isolation for cultures) from the environment when isolating a species into a monoclonal culture, leaving loads of nutrients available to cells and plenty of space to grow without predator grazing. Additionally, some diatom cells thrive under culture conditions by removal of competitors, leaving huge space for cells to grow and opportunity to use all nutrients and light available (De Jong et al., 1984 and references therein).

Furthermore, cells of various *Entomoneis* species had been noticed, with previous studies, microscopically in field samples in the southern, middle and northern Adriatic Sea, but were mostly identified as small *Amphiprora* spp. or even *Licmophora* spp. due to their unusual twisted appearance and light silification (Bosak and Marić Pfannkuchen, *personal communication*). The generally complex three–dimensional structure and light silification of planktonic *Entomoneis* frustules requires detailed light and electron microscope observations of the valve and girdle elements for accurate species identification. This is usually avoided (and not possible under LM only) in general ecological studies where quantitative methods such as Utermöhl (Lund et al., 1958) are main tools to estimate phytoplankton abundances. Morphological differences between *Entomoneis* species in this thesis are well supported with phylogenetical separation with the exception of *E. pusilla* and *E. gracilis*. The last two are well distinguished with morphological features. *E. pusilla* is smaller, more silicified, with lanceolate valves and scalpeliform valve apices, with distinguishable narrow dash-like perforations within striae, specific copulae structure and hooked terminal raphe endings. On the other hand, *E. gracilis* cells are bigger, generally narrower in girdle view, with narrow lanceolate valves and broad scalpeliform apices, and with round poroids in striae, while girdle elements do not resemble *E. pusilla* at all. However, these two are phylogenetically positioned in the same clade, suggesting they should be described as one species. This particular example is good for applying multiple species concepts to correct species identification, as here both PSC and MSC need to be considered together to notice species delineation.

Publications **III-VI** contribute to biodiversity knowledge in the southern Adriatic Sea with the characterization of one known diatom species, sporadically present in the Adriatic Sea (*Pseudo-nitzschia mannii*) and two yet undescribed new species of tychopelagic pennate diatoms (*Haslea* sp.) and pico coccoid green Trebouxiophyte (*Picochlorum* sp.). All three species were characterized with morphology and phylogeny (for *Haslea* sp. publication including phylogenetic data is in preparation), and with additional biotechnological potential characterization through observation of cells in cultivated conditions with different methods and measurements of pigment and lipid content. Publication **III** generally highlights the usage of multiple tools in identification of a species, modelled on publications **I** and **II**, while publications **IV-VI** provide stepping stones for future research on these interesting organisms. Plenty of biotechnological studies are conducted at the moment for the genera *Haslea* and *Picochlorum*, and for which basic characterization has been done (De la Vega et al., 2011; Dahmen et al., 2014; Gastineau et al., 2014; Tran et al., 2014; Falaise et al., 2016; Prasetiya et al., 2017). These are the examples of biodiversity studies (where the primary goal is to identify species) favouring biotechnology that represents future investigation potential.

The publications within this doctoral thesis altogether contribute to the second thesis question: *"Is the Adriatic Sea a good model for studying shifts in diversity in the plankton communities due to ongoing climate changes?"* with great discoveries of biodiversity of planktonic pennate diatoms, green pico algae and a first glimpse on composition of total PEs community. Ecological aspects of species were not investigated in detail, so the second part of the question may not be answered directly, but hypothesised, as ongoing climate change depend on many parameters that need to be examined over a longer period. The Adriatic Sea represents specific habitat in accordance to its geomorphology, position, connectivity with the Mediterranean Sea and Atlantic Ocean, physical forcing and specific BiOS concept of circulation, and as the whole Mediterranean Sea, is a marine biodiversity hotspot (Coll et al. 2010).

In the light of studying diatoms, investigations in the Adriatic Sea are not scarce, but most of the studies put bigger effort in taxonomy and ecology of centric planktonic diatoms than to the pennate ones (Burić et al., 2007; Viličić et al., 2009; Bosak et al., 2012; Bosak and Sarno, 2017; Čalić et al., 2017). According to the phytoplankton checklist made by Viličić et al. (2002), there are 518 taxa recorded (330 pennates and 174 centric diatoms). However, a previous checklist made by Revelante et al. (1985) had total of 296 diatom taxa listed from northern Adriatic Sea, which were not found later in the area (Viličić et al., 2002). This huge discrepancy between two checklists insinuates various reasons such as: i) the diatom flora changed during the years; ii) the earlier research was done more carefully and detailed; iii) the earlier research had more samples including tychoplanktonic species found in plankton due to the mixing and upwelling of benthos; iv) the later research had limited numbers of samples and had investigated phytoplankton in less detail. Additionally, most of the studies on centric and pennate diatoms in this area relied on morphology and ecology (Caroppo et al., 2005; Ljubešić et al., 2011; Marić et al., 2011; Arapov et al., 2017). There are only a few studies performed in the northern and middle Adriatic Sea combining both morphology and phylogeny in detection and correct taxonomical identification of planktonic centrics and pennates (Kooistra et al., 2008; Pletikapić et al. 2011; Godrijan et al., 2012; Penna et al., 2012; Bosak et al., 2015; Marić Pfannkuchen et al., 2018). Low numbers of publications based on this combined approach, of which most of them deal with *Pseudo-nitzschia* species, leave a great knowledge gap for the complete picture of diatom biodiversity in the Adriatic plankton. As mentioned in the *Introduction*, planktonic pennate diatoms are globally underappreciated in comparison to centrics, with an exception of few pennate diatom genera that are forming specific colonies in the plankton (eg. *Pseudo-nitzschia*, *Thalassionema*, *Asterionellopsis*). Nevertheless, single-celled planktonic pennate diatoms are also frequently found in the plankton, but are mostly in low numbers or are small and lightly silicified, leaving scientist an impossible identification task based on morphology only.

Publication **VII** contributes to biodiversity knowledge on picoeukaryotic plankton worldwide and especially in the Adriatic Sea. General results of this publication showed extremely low number of sequences attributable to PPEs, with HPEs dominating the community, what is congruent to other oligotrophic environments, such as the Red Sea, the Atlantic and Pacific Oceans (Shi et al., 2009, Acosta et al., 2013, De Vargas et al., 2015, Pernice et al., 2015, Pearman et al., 2017). In an oligotrophic environment, high abundances of heterotrophs in picofraction are consistent with Margalef's mandala, which emphasizes that in highly turbulent and low nutrient environments, picocyanobacteria such as *Prochlorococcus* and *Synechococcus* are the main primary producers (Glibert, 2016). Additionally, apparent oxygen utilization (AOU) measurements reported by Babić et al. (2018) in the same investigated area are congruent with the HTS findings that the southern Adriatic Sea has respiration as the main process (within microbial loop) instead of primary production. Here we have an example of biodiversity enabling ecology, which with a multidisciplinary approaches such as the ones shown in Babić et al. (2018) with physical, chemical and biological properties of the water column are screened, successfully described the studied ecosystem as a natural laboratory important to be further investigated.

The third question of this thesis *"What is the possibility of isolating new strains with potential in biotechnology?"* also generates wide spectre of possible answers and methodology in research, by which question can be completely answered. However, in this thesis we just "scraped the surface" into the biotechnology research of newly isolated strains of planktonic pennate diatoms and green algae (Publications **IV-VI**). Generally, diatoms are of biotechnological interest since they produce unsaturated fatty acids which, in combination with their amphorous silica cell walls, represent a good basis for bio-mineralization processes that can result in nano-technological findings which are of great potential for today's science (Kroth, 2007). In addition to diatoms, other microalgae, among them most prominently green microalgae, such as *Chlorella* or *Tetraselmis*, are widely used in the field of biotechnology (Lavens, 1996). A great variety of compounds are obtained from microalgae for industrial applications such as dyes, antioxidants, emulsifiers, aminoacids, fatty acids (mostly omega 3 and omega 6), moisturizing for cosmetic, bio-combustible and environmental applications, etc. (De-Bashan et al., 2004; Spolaore et al., 2006; Williams and Laurens, 2010). Additionally, it is important to mention that the synergy between molecular characterization and genetic modifications of algae together with biotechnological experiments can have a great potential to the industry (example of diatom *Phaeodactylum* sp., Walker et al., 2005). Although we had no problems to identify potentially biotechnologically important genera such as *Haslea* and *Picochlorum* with morphology and phylogeny only, a wide variety of species demands combination of biochemical, physiological and morphological characters to create a taxonomic classification (Dayan et al., 2010). Characterizations of isolated strains in publications **III-VI** are congruent with methodology of recent studies (Henley et al., 2004; Amato and Montresor, 2008; Ruck and Theriot, 2011; Gastineau et al. 2014, 2016). Likewise, these characterizations are important in the light of increasing visibility of those genera/species in the context of sequences and information storage into open databases such as GenBank, as well as in the context of providing good quality images of voucher strains (Pniewski et al., 2010; Ruck et al., 2016; Witkowski et al., 2016).

Species concept of this thesis

Previously mentioned and characterized as the most difficult question of this thesis „*Where is the phylogenetical border between species and genus in picoeukaryotes and planktonic pennate diatoms? Does the current knowledge of species/genus border adequately reflect the use and availability of new genetic markers in picoeukaryotes and planktonic pennate diatom research and microscopy in classical morphology?*" is discussed with usage of multiple species concepts through thesis publications. Within publications **I, II, III** and **VI**, and for publication **IV** yet unpublished molecular data, specific gene markers were used to identify isolated marine diatoms and green algae, which resulted in seven new species descriptions and confirmation for two more new species yet to be described (*Haslea* sp. and *Picochlorum* sp.). Specific gene markers were selected according to the newest available literature for those genera (Henley et al., 2004; Ruck and Theriot, 2011). However, as the field of phylogeny is evolving fast, especially considering diatoms and pico green algae, this means that the introduction of more gene markers into phylogenetic analyses will be necessary (Theriot et al., 2015; Barcytė et al., 2017). Using only one genetic marker for species identification, as well as looking at morphology only, can often mislead correct identification of species, expressing the need on taxonomic revisions and species transfer from one genus to another (Henley et al., 2004; Theriot et al., 2009, 2010, 2011; Sterrenburg et al., 2015). Generally, many studies showed that concatenation of several genes sums up the collective signal of several genes, (Zhang et al., 2008; Theriot et al., 2015; Medlin, 2016; Li et al., 2017; Pinseel et al., 2017; Barcytė et al., 2017). Most scientists today believe that a 'good' species should be genotypically and phenotypically distinct from the others, where the 'phenotype' is a much more inclusive category than morphology alone.

We need to emphasise the fact that the diatoms and green algae within this thesis besides genera *Pseudo-nitzschia* (meaning genera *Entomoneis*, *Haslea* and *Picochlorum*) in the Adriatic Sea were investigated using molecular tools for the first time. They are most probably omnipresent species in the Adriatic Sea, but have so far been neglected or misinterpreted in the field samples. However, it is hard to tell with certainty if all described species of *Entomoneis* represented in publications **I** and **II** were indeed noticed in field samples over the years, as no steady monitoring program was introduced and performed in the middle and southern Adriatic

Sea. This problem had been addressed before, in the case of small multipolar diatom genus *Bacteriastrum*, also lightly silicified diatom, found in plankton in large chain colonies (Godrijan et al., 2012). Through years, one particular species, *B. jadranum* had been identified as small *Thalasiossira* (Bosak, *personal communication*), but Godrijan et al. (2012) proved and described new species combining morphology and phylogeny. Here it is important to emphasize consequences in misinterpretation of a species in absence of better identification tools, as in this case would have been the usage of electronic microscopy, and of course molecular tools. Additionally, usage of multilayer approach in taxonomical identification allows us to uncover the existence of cryptic and pseudocryptic species that are discovered every day (Beszteri et al., 2005b; Amato et al., 2007; Kooistra et al., 2008; Ellegaard et al. 2008; Pinseel et al., 2016; Gaonkar et al., 2017). Cryptic species are defined as morphologically identical but genetically distinct entities, while the pseudo-cryptic ones present, besides genetic divergence, also minor ultrastructural differences only detectable by very accurate morphological analyses (Mann & Evans, 2007). Although the discovery of seven new *Entomoneis* species in marine plankton can resemble a case of 'cryptic' diversity, combination of morphological and molecular tools for species description provided appropriate resolution to conclude there is nothing hidden or cryptic among those species. Crypticism within this doctoral thesis was addressed in a case of characterization of *Pseudo-nitzschia mannii* (Publication **III**), an already known cryptic species, which is hardly distinguishable from similar *P. calliantha* with fine ultrastructural arrangement of the poroid sector and slightly wider cells (Amato and Montresor, 2008). We managed to correctly identify this species thanks to molecular identification according to three gene markers, which evokes the necessity of combining methods in species characterization, and that neither species can be assessed by just one species concept. Within publications **I** and **II** the interesting situation arose when using single gene phylogeny to delineate species – that according to *psb*C and SSU gene phylogenies the genus *Entomoneis* was paraphyletic. This is particularly important for understanding the border between genera and species, as a different number of newly described species would have been discovered if we had used just SSU or just *psb*C gene, and ignored morphological differences/similarities. Interestingly, *rbc*L gene phylogeny resolved the genus *Entomoneis* as monophyletic in single gene phylogeny and in concatenated versions, which goes in favour of many studies evoking this gene marker to be used as species delineation marker in taxonomic studies and in metabarcoding approaches (MacGillivary and Kaczmarska 2011; Zimmermann et al., 2015; Vasselon et al., 2017). In comparison to *Entomoneis* species described in publications **I** and **II**, here it is worth mentioning that *Pseudo-nitzschia mannii* is much harder to isolate for monoclonal cultures, mainly because its occurrence in the Adriatic Sea is sporadic, its abundances are lower, and under LM, scientist could never be sure that it is indeed *P. mannii* to be isolated for culture. This can be very important in correct identification of species since many diatoms cannot be isolated into cultures, which is the main reason they remain undetected or misinterpreted. In case of a three gene phylogeny in *P. mannii*, we could not combine all three genes into a single matrix due to statistically unsupported heterogeneity test performed on SSU, ITS and LSU sequences and different evolutionary rates of each gene marker. However, placement of *P. mannii* strain from Telašćica Bay was always the same, indicating this genus has better resolution, which can be attributed to more species described and more strains isolated for cultures and sequences.

All three publications state the importance of using both morphology and phylogeny in correct taxonomical identification of a species, and on the other hand give space to hypothesise population separation by isolated and specific environments such as middle and southern Adriatic Sea. Additionally, all three publications suggest the usage of multiple gene markers in identification of a species, along with single gene phylogenies can produce inconclusive results. In favour to separation by the isolated environment hypothesis goes the fact that *Entomoneis* ribotypes were not observed in large metagenomic datasets such as ones from Tara Ocean or Ocean Sampling Day (Malviya et al. 2016; https://www.ebi.ac.uk/metagenomics/projects/ERP009703). On the other hand, within results obtained by high-throughput sequencing of the 18S rRNA amplicon in pico-fraction (publication **VII**) some sequences belonging to the genus *Entomoneis* were identified. Geomorphological positioning and physical forcing in the southern Adriatic Sea could affect *Entomoneis* cells so they can be retained and 'isolated' in that area, leading to genetic separation of the Adriatic population from other known *Entomoneis* species. However, yet undescribed *Entomoneis* strains from Tasmania, California and Arabian Sea that grouped as sister species with *E. gracilis*, *E. vilicicii* and *E. umbratica* (publication **II**) indicate that this hypothesis is very unlikely. Regarding *Pseudo-nitzschia mannii*, early steps in possible species speciation can be fostered by the highly indented coastline of the eastern Adriatic Sea, more specifically, Telašćica Bay from which cells were isolated. The geological and oceanographic characteristics of the Telašćica Bay allow scenarios in which cells of *P. mannii* could be retained and separated from the open Adriatic Sea, and consequently lead to successful genetic drift. Nevertheless, this can also remain a hypothesis, as hard evidence for speciation were not yet clarified, and as phylogenetic placement of Adriatic *P. mannii* together with other strains of *P mannii* isolated from all over the world can overrule it. In the context of global studies, publications **I-III** are congruent with newest species descriptions/characterizations in context of using multiple gene phylogenies and detailed morphological analyses (Theriot et al., 2015; Ruck et al. 2016; Li et al., 2017; Pinseel et al., 2016, 2017).

Kociolek and Williams (2015) emphasised the criteria under which a diatom genus must be defined, and the most important feature is that genus is monophyletic. This 'rule' comes from cladistics, a theory of systematics focused on defining phylogenetic relationships among living organisms in a way of taking into account only those shared characteristics between organisms which can be deduced to have originated in the common ancestor of a group of species during evolution, not those arising by convergence. Therefore, in spirit of cladistics, that seeks certain characters common for organisms, monophyletic groups should have unique characters, and should represent a specific part of evolutionary history: they are collections of species that are more closely related among themselves rather than to anything else (Kociolek and Williams, 2015). In case of *Entomoneis* species, monophyletic origin could not be confirmed if single gene phylogenies were used. This means that to correctly describe a diatom genus, concatenated phylogeny derived from three genes was obligatory to use in publications **I** and **II**. When criteria for erecting a genus are not followed as supposed to, problems in lower taxonomic categories such as species can follow, leading to unnecessary description of more genera, as it previously happened within the large genus *Eunotia* (Wetzel et al., 2010, and references therein). Regardless of the organism (diatoms, green algae, etc.), phylogenetic approach to species delimitation has some serious limitations. Delimiting species boundaries among closely related lineages often requires a range of independent data sets and analytical approaches or even empirical, sequence-based species delimitation approaches (Wei et al., 2016). DNA based taxonomical approaches can improve our estimations for inter- and intraspecific genetic variation, but thresholds are difficult to establish. Probably the most known (and controversial) stated threshold for genetic divergence is by Herbert et al. (2004) who stated the 10× rule in metazoans or 3% divergence between inter-species (Smith et al. 2005). The rule claims that a genetic variation of 10× the average intraspecific difference indicated a new species in metazoans, or that different species must diverge at least in 3% of their sequences (Herbert et al., 2004; Smith et al. 2005). Such threshold-based approaches are known for many organism groups, as well as for diatoms (Zimmermann et al., 2011). In his study, Zimmermann et al. (2011) tested various PCR primers of V4 region of 18S rRNA gene, and concluded which are the best to use according to calculated p-distances among species belonging to the same genus. V4 region (390-410 bp long) is suitable for species discrimination as it holds many variable character sites, inversions, insertions and deletions, resulting in a highly concentrated information content on a very short fragment (Alverson et al., 2006).

As mentioned in the *Introduction*, *Species concepts in protistology*, many species concepts can be applied to both diatoms and PEs. Probably the most important ones are the MSC and the PSC. All publications in this thesis are dealing with correct identification according to certain or combined concepts. Within publications **I-III** both MSC and PSC are applied when species were identified, and we can say for sure we would not have correct identifications if we did not combine these concepts. A good example of how leaning on just one (MSC) concept in species description can lead to incorrect results gave Sterrenburg et al. (2015) who described, based on morphology, seven new species belonging to the diatom genus *Haslea*; while Li et al. (2017) proved with combined PSC and MSC that two of those species belong to the genus *Navicula*. Another good example is the genus *Picochlorum*; Henley et al. (2004) erected this new genus from previously described genus *Nannochloris* by combining three species concepts: BSC (reproductive isolation), MSC (specific morphological characters) and PSC (phylogenetic placement of species based on two gene markers) and have transferred several species to the newly described genus. By the same technique, hundreds of species of algae are transferred into new combinations and new genera by scientists in publications. However, evidences on evolving strategies for better species delimitation are visible in every species concept and are reported daily: (i) microscopic and sequencing abilities and image quality are profoundly enhanced, so scientist can observe some characters/taxa we previously ignored or were not able to detect; (ii) reproductive isolation experiments among species are enhanced with cultivation success of previously uncultivated taxa; and (iii) molecular data are added daily to the open databases that could provide us with better species resolution.

CONCLUSIONS

The most important conclusions, which have arisen from the publications that comprise this doctoral thesis, are:

1. Biodiversity of pennate planktonic diatoms and PEs are to a great deal unknown, both globally in world oceans, and locally in the Adriatic Sea. This thesis contributed to the knowledge on these marine microbes, providing us with stepping stones for future research regarding taxonomy, molecular biology and biotechnology.

2. Species concepts for protists are diverse, and this thesis tested the most important of them on the studied diatom and green algae genera. This thesis highlights that a sound way for defining a genus/species is to combine MSC and PSC, especially when several gene markers are considered.

3. The Adriatic Sea, especially the southern and middle part can be considered a biodiversity 'hotspot' and a natural laboratory for investigations on species taxonomy and ecology.

4. This thesis provided a stepping stone in biotechnology studies on algae, an evolving field of science, and completely neglected field of research in Croatia and the Adriatic Sea.

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CURRICULUM VITAE

Maja Mucko (Mejdandžić) was born in Uskoplje, Federation of Bosnia and Herzegovina, May 2nd 1991. She finished elementary, musical elementary and high school in Knin, Croatia, and continued education in Zagreb, Croatia, at University of Zagreb, Faculty of Science, Department of Biology. She finished undergraduate studies in biology in 2012, gaining a title bachelor of biology (univ. bacc. biol.), after which finished graduate programm in ecology and environmental protection in 2014, gaining a master of ecology and environmental protection title (mag. oecol. et prot. nat.). Her employment at the University of Zagreb, Faculty of Science, Department of Biology started in January 2015 as expert associate in science and education, and since January 2017 she is employed in the same institution as research assistant. She started her Ph.D. programm at the same University in November 2014, and have finished in July 2018, gaining a title of doctor of Philosophy (Ph.D.). During her Ph.D. she was working in the frame of the Croatian Science Foundation project “Bio-tracing Adriatic Water Masses” (BIOTA). She published 14 scientific and professional papers and have participated in 16 scientific conferences (13 international and 3 domestic) with 26 conference papers. Additionally, she participated in educational work as a teaching assistant on one undergraduate, one graduate and one field course for students. During the past three years, she has been trained several times abroad and in Croatia, attending classes and seminars on the Next Generation Sequencing and statistical processing of data in the programming language R. In addition to her scientific work, she is also active in professional activities, and since 2014 she is a secretary of the Croatian Botanical Society. She has held two popular scientific lectures for the general public.

SCIENTIFIC ID in Croatian Scientific Bibliography: 359773

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Mejdandžić M, Cetinić I, Mihanović H, Vilibić I, Ljubešić Z (2017, January) Picoeukaryotic plankton composition revealed by high-throughput sequencing: first data for the Adriatic Sea. In The 15th Symposium on Aquatic Microbial Ecology.

Bosak S, **Mejdandžić M**, Piltaver IK, Petravić M, Gligora Udovič M, Ljubešić Z (2017, January) Diversity of pennate diatoms in the plankton of the southern Adriatic Sea. In 23rd Nordic DiatomistsMeeting 2017.

Mejdandžić M, Bosak S, Orlić S, Gligora Udovič M, Peharec Štefanić P, Špoljarić I, Mršić G, Ljubešić, Z (2017, January) Hidden diversity of planktonic *Entomoneis* species. In 11th Central European Diatom meeting.

Bosak S, **Mejdandžić M**, Wetzel CE, Peharec Štefanić, P, Ljubešić, Z (2017, January) Observations of planktonic *Nitzschia* and *Navicula* from the South Adriatic Sea. In 11th Central European Diatom meeting.

Vugrin M, **Mejdandžić M**, Bosak S, Mihanović H, Dupčić-Radić I, Godrijan J, Orlić S, Ljubešić Z (2016, January) Taxonomy and spatial distribution of coccolithophors in the south Adriatic: winter aspect. In 5th Croatian Botanical Symposium.

Raos P, Bosak S, **Mejdandžić M**, Ljubešić Z (2016, January) Microphytoplankton winter diversity in the South Adriatic Sea. In 5th Croatian Botanical Symposium.

Mejdandžić M, Bosak S, Gligora Udovič M, Peharec Štefanić P, Kružić P, Špoljarić I, Mršić G, Orlić S, Ljubešić Z (2016, January) *Entomoneis* species in the Adriatic Sea. In 5th Croatian Botanical Symposium.

Mejdandžić M, Bosak S, Orlić S, Ljubešić Z (2016, January) A new marine planktonic *Entomoneis* species from the Adriatic Sea. In 10th Central European Diatom Meeting.

Bošnjak I, Petrić I, Cetinić I, Bosak S, **Mejdandžić M**, Kružić P, Mihanović H, Miloslavić M, Lučić D, Ljubešić Z (2016, January). Ocean optics meets taxonomy: case study in Southern Adriatic Pit. In EMBO EMBL Symposium: A New Age of Discovery for Aquatic Microeukaryotes.

Mejdandžić M, Orlić S, Bosak S, Bošnjak I, Cetinić I, Peharec Štefanić P, Sesar T, Gašparović B, Ljubešić Z (2016, January) Charactrization of photosynthetic picoeukaryotes from the Southern Adriatic Pit. In EMBO| EMBL Symposium: A New Age of Discovery for Aquatic Microeukaryotes.

Malešević N, Koletić N, **Mejdandžić M**, Blinkova M (2015, January) Preliminary results of a biodiversity of Blue-green algae (Cyanobacteria) along the karstic river Cetina. In 6th Balkan Botanical Congress.

Mejdandžić M, Malešević N, Koletić N, Nemet M, Kralj Borojević K (2015, January) Phytobenthos and aquatic vegetation as biological quality and habitat description elements along submountain karstic river. In Croatian 12th Biological Congress.

Bošnjak I, Mihanović H, Cetinić I, Kružić P, Bosak S, **Mejdandžić M**, Ljubešić Z (2015, January) Spatial distribution of biooptical and thermohaline characteristics in NW part of Southern Adriatic pit. In 12th Croatian Biological Congress.

Bosak S, **Mejdandžić M**, Bošnjak I, Godrijan J, Ljubešić Z (2015, January) Taxonomy and spatial distribution of phytoplankton in the south Adriatic; winter aspect. In 12th Croatian Biological Congress.

Ljubešić Z, Bosak S, Bošnjak I, **Mejdandžić M**, Mikac I, Cetinić I (2015, January) Patchy distribution of phytoplankton pigments in South Adriatic oligotrophic environment-winter 2015. In 6th Balkan Botanical Congress.

Mejdandžić M, Mihanović H, Šilović T, Henderiks J, Šupraha L, Polović D, Bosak S, Bošnjak I, Cetinić I, Olujić G, Ljubešić Z (2015, January) Biomarker pigment divinyl chlorophyll *a* as a tracer of water masses?. In 6th European Phycology Congress.

Mejdandžić M, Malešević N, Nemet M, Kralj Borojević K, Gligora Udovič M (2015, January) Phytoplankton and phytobenthos as biological quality elements along karstic river. In 9th Central European Diatom Meeting.

Mejdandžić M, Ljubešić Z, Ivanković T, Pfannkuchen M, Godrijan J, Hrenović J (2014, January) Colonization of diatoms and bacteria on artificial substrates in the marine environment. In 8th Central European Diatom Meeting.

Koletić N, Hanžek N, Polović D, **Mejdandžić M**, Linardić M, Slana A (2012, January) Contribution to the Knowledge of Macro-algae of the Coastal Area of the Island of Hvar. In BALWOIS 2012.

SCIENTIFIC PROJECTS

(2015-2017) Collaborator on UIP-2013-11-6433 (BIOTA – Bio-tracing Adriatic Water Masses)

(2016-2020) Collaborator on COST Action DNAqua (Developing new genetic tools for bioassessment of aquatic ecosystems in Europe CA15219)

(2018-2023) Collaborator on UIP-2017-05-5635 (TurtleBIOME - Loggerhead sea turtle (*Caretta caretta*) microbiome: insight into endozoic and epizoic communities)

SCIENTIFIC COLLABORATIONS

Teofil Nakov and Elizabeth Ruck (University of Arkansas, Fayetteville, USA): *Entomoneis* species in the Adriatic Sea – phylogenetic analyses and paper publication

Daniel Vaulot (Station Biologique de Roscoff, Roscoff, France): *Picochlorum* species in the Adriatic Sea – advices for data analyses, paper publication

Romain Gastineau (University of Szczecin, Szczecin, Polland): *Haslea silbo* sp. nov.; description of two new

Haslea species, of which one was isolated from the Adriatic Sea, paper publication

Laurence Garczarek (Station Biologique de Roscoff, Roscoff, France): *Prochlorococcus* ecotypes in the Adriatic Sea revealed by petB gene analysis – advices in data analysis, data analysis with internal database

Sandi Orlić (Institut Ruđer Bošković, Zagreb, Croatia): Molecular identification of Adriatic diatoms, picoeukaryotes and bacteria: learning of basic molecular methods, usage of molecular biology equipment

WORKSHOPS, SCIENTIFIC TRAINING AND ORGANIZING EXPERIENCE:

(2018) Workshop ″Molecular tools in algology″ in organization of Croatian botanical society

(2017) School on ″Using R and Stats in Next Generation Sequencing data″ in organization of Exaltum Ltd., Zagreb, Croatia

(2017) COST Action CA15219 ('DNAqua-Net')- Developing new genetic tools for bioassessment of aquatic ecosystems in Europe, workshop of WG2, development of diatom indices, Zagreb, Croatia

(2017) COST Action CA15219 ('DNAqua-Net')- Developing new genetic tools for bioassessment of aquatic ecosystems in Europe, workshop of WG2, development of diatom indices, Sarajevo, Bosnia and Herzegovina

(2017) Analysis of Gene Expression and Regulation on Several Levels seminar in organization by Promega and GeneTeam

(2017) Next generation sequencing in medical practice, seminar in organization by Kemomed d.o.o. (KBC Zageb, Croatia)

(2016) Workshop on project AMBIOMERES (Appearance and interaction of biologically important organic molecules and micronutrient metals in marine ecosystem under environmental stress (AMBIOMERES IP-11- 2013-8607)), Institute Ruđer Bošković, Zagreb, Croatia

(2016) ‘’12th Summer Course on Marine Ecological & Evolutionary Genomics’’ held by Station Biologique de Roscoff, Roscoff, France

(2016) ‘’Applications of scanning electron microscopy, energy-dispersive spectroscopy and wavelength-dispersive spectrometry’’ held by dr. sc. Suzana Erić from University of Belgrade; University of Zagreb, Faculty of Mining and Geology, Zagreb, Croatia

(2016) Member of technical support at 5. Croatian botanical symposium, Primošten, Croatia

(2015) Next Generation Sequencing in daily practice Seminar held at University of Maribor, Faculty of Medicine in organization by Kemomed d.o.o., Maribor, Slovenia

(2015) Scientific training at Ruđer Bošković Institute, use of SEM, cultivation of marine phytoplankton cells, sedimentation and abundance estimation of phytoplankton – Marine research center, Rovinj, Croatia

(2015) Scientific training at Alfred Wegener Institute (AWI), Bremerhaven, Germany

(2015) Workshop held by dr. sc. Marko Košiček - ″How to present science in the media″, University of Zagreb, Zagreb, Croatia

(2015) Member of technical support at 12. Croatian botanical congress, Sveti Martin na Muri, Croatia

(2015-up to date) Secretary and treasurer of Croatian botanical society

(2014) Member of technical support team at 8th Central European Diatom Meeting (Zagreb, Hrvatska)

(2014) International research and educational camp ″Grabovača″ in organization of Croatian student biology association BIUS

(2013-2014) Head of Algology section in Croatian Biology Student Association (BIUS)

(2013-2014) Organization of international research and educational project ″APSYRTIDES″ Cres, Croatia, Biology Student Association (BIUS)

(2011) Research and educational camp ″Moslavačka Gora″ in organization of Croatian Biology Student Association (BIUS)

(2011) International research and educational camp ″Hvar 2011″ in organization of Croatian Biology Student Association (BIUS)

(2010-2012) Popular scientific manifestation ″Biology Night″ at University of Zagreb, Faculty of Science, Biology Department