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A blue-pigmented hasleoid diatom, *Haslea* sp., from the Adriatic Sea

by

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Abstract

We present the first report and description of the pinnate diatom *Haslea* sp. from the northeastern Adriatic Sea, Croatia, producing a blue pigment. This organism is very similar to the well-known *Haslea ostrearia*, the first described "blue" diatom producing marennine, i.e. the pigment involved in the greening of oysters, and recently described *H. provincialis*. However, the Croatian diatom slightly differs from other *Haslea* species in its morphology and 18S rRNA sequence. The discovery of *Haslea* sp. from Croatia confirmed the possible existence of more species among the representatives of blue *Haslea* species, as previously assumed. The discovery of several genetically distinct populations of *Haslea ostrearia*, new species *H. karadagensis*, *H. provincialis* and *Haslea* sp. from Croatia, suggests that species richness in the group of "blue" diatoms is probably underestimated and still more new blue diatoms remain undiscovered. This also raises questions about previously published reports and observations of *Haslea* distribution in the Mediterranean Sea whether these organisms really belong to *H. ostrearia*.

Key words: Bacillariophyceae, Mediterranean Sea, taxonomy, 18S rRNA

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Introduction

Diatoms are usually referred to as golden-brown microalgae, due to the color of their plastids and pigment composition, mainly carotenoids (fucoxanthin, diadinoxanthin, diatoxanthin), which mask chlorophylls *a* and *c*.

The pennate marine diatom, *Haslea ostrearia* (Gaillon) Simonsen, is a tychopelagic or benthic organism, remarkable for its ability to produce a water-soluble blue pigment, commonly called marennine that accumulates mainly in the apical regions of the cells. In oyster ponds of western France, where *H. ostrearia* regularly dominates, marennine is released into the seawater and is responsible for the "greening" of oysters. This phenomenon, having a locally significant economic impact, has long been known (Sprat 1667). The involvement of a microscopic alga was recognized later but still a long time ago, and the organism was first described as *Vibrio ostrearius* (Gaillon 1820; Dyer 1877), then as *Navicula ostrearia*, and finally transferred to the genus *Haslea* (Simonsen 1974).

At present, the genus *Haslea* comprises more than 20 species that may show significant differences in their morphology, ecology or physiology. For example, *Haslea wawrikae* is a planktonic diatom with very long and acute apices, and *Haslea gigantea* is a mesoplanktonic species with a cell length of up to 500 μm (Simonsen 1974). Most other *Haslea* species are known for being benthic organisms, and *Haslea crucigera* is a tube-dwelling species.

Diatoms with blue tips, recorded as *H. ostrearia*, have also been reported from almost all seas and oceans, in both the Northern and Southern Hemispheres, either from direct observations or through the greening effect on bivalves. In Northwestern Europe, records come from the coasts of Great Britain (Sprat 1667; Hustedt & Aleem 1951), Belgium (M'harzi et al. 1998), Norway (Hendey 1964), Sweden (Wulff et al. 2000), and the Baltic Sea (Snoeijs & Kasperoviciene 1996). In the northern Atlantic Ocean, "blue diatoms" have been found in the Canary Islands (SCCAP, Gert Hansen), different places along the North American coast (Kennett & Hargraves 1991) and possibly in Honduras (Grunow 1877). In the northern Pacific Ocean, *H. ostrearia* has been reported from the San Juan Islands (Hardy 1973) and from Japan (Ranson 1937). In the Southern Hemisphere, diatoms identified as *H. ostrearia* have been observed in the Indian Ocean (Simonsen 1974) and reported from eastern Tasmania (Volkman et al. 1994).

A recent approach combining SEM, molecular barcoding and reproductive biology has resulted in the discovery of *H. karadagensis* Davidovich, Gastineau & Mouget, a new species of bluish diatoms in the Black Sea (Gastineau et al. 2012). In July 2009, a strain of diatoms with blue apices was isolated from the French Mediterranean shores at Boulouris, and kept in the Scandinavian Culture Collection of Algae and Protozoa (SCCAP) under the name of *Haslea provincialis* (Gastineau et al. 2016). These records suggest that the microalgae producing a blue pigment have a worldwide distribution.

In this paper, we report on a possibly new species from the genus *Haslea* from the Croatian shore of the Adriatic Sea and its morphological as well as molecular identification.

Materials and methods

Study area and sampling site

In August 2012, diatoms with dark bluish apices were found in Saharun Bay on the island Dugi Otok, near the settlements of Božava, located in the northeastern Adriatic Sea (Croatia) (Fig. 1).

Samples were collected from stagnant pools approximately 2–7 m from the seashore harboring ca. 0.3-cm-thick microbial mat, using a sterile scalpel. *Haslea* sp. densely covered the upper part of *Padina* sp. at a depth of 5–10 cm (Fig. 2), usually growing in solitary cells.

Subsamples for DNA analysis were preserved in guanidine thiocyanate solution (GES) in 2 ml Eppendorf tubes. GES-solution included 5 M guanidine thiocyanate, 100 mM EDTA with pH 8.0 and 3.4 mM N-lauroyl sarcosine (Abed et al. 2006). Subsamples for further microscopic analysis and morphometry were preserved in 3% formaldehyde solution in environmental water.

Light microscopy and morphometric analyses

Light-microscopic analyses and photomicrographic documentation were carried out using transmitted light, phase contrast, Nomarski differential interference contrast (DIC), illumination and epifluorescence.

Morphometric analysis of cell dimensions was carried out from in-scale camera Lucida projections and scanned photomicrographs using Sigma Scan Image software (Jandel Scientific, Sausalito, CA) with its statistics package. Differences in dimensions were expressed as mean ± standard deviation (n = 30–50).

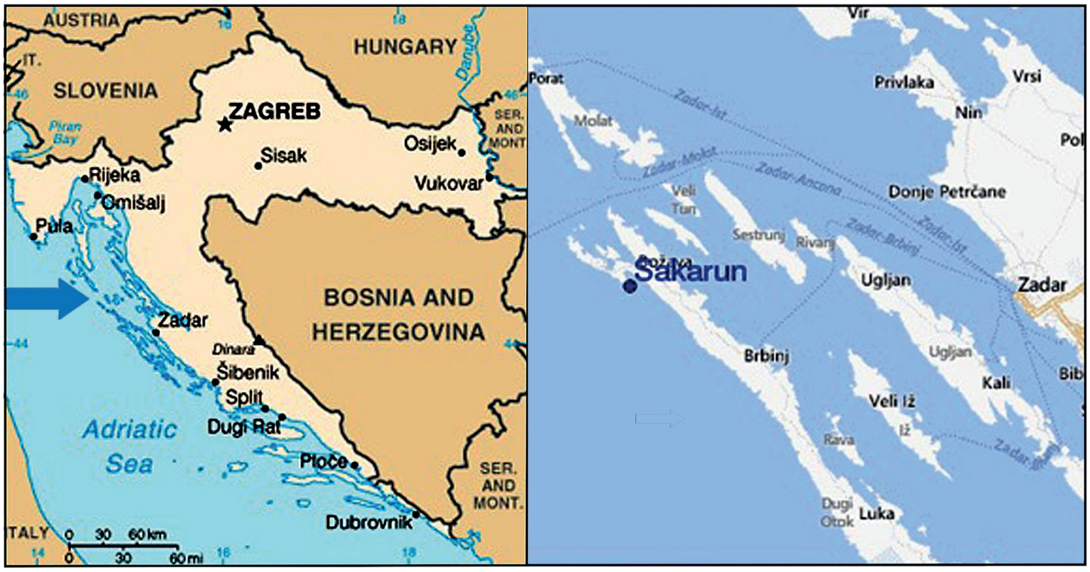

Figure 1

Sampling site in Croatia, Dugi Otok island at Saharun Bay, the Adriatic Sea (Google maps)

Molecular analyses

The total DNA was extracted from 1 ml of the *Haslea* guanidine thiocyanate suspensions using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, USA) and following the procedure described by Palinska et al. (2006). Five freeze (liquid nitrogen) and thaw (water bath at 60°C) cycles were applied for maximum yields. The 18S ribosomal RNA (rRNA) gene was amplified with the diatom-specific primers described by Zimmermann et al. (2011). The PCR reaction of 50 μl contained 1 × RED Taq PCR Buffer, 200 μM of each deoxynucleotide, 100 μg BSA, 250 ng of each oligonucleotide primer, 2.5 U of RED Taq DNA polymerase (Sigma-Aldrich), and 5µl of DNA extract. The presence of purified PCR products (QIAquick PCR Purification Kit, Qiagen, Germany) was detected by standard agarose gel electrophoresis (1.5%) and ethidium bromide staining.

DNA samples were directly sequenced in both directions by a commercial sequencing laboratory, using the same primers as for amplification. The combination of D512for 18S and D978rev 18S primers (Zimmermann et al. 2011) enabled the amplification of a 466 bp fragment.

The obtained 18S rRNA sequence was aligned with selected GenBank entries using clustalW (http://www.ebi.ac.uk/clustalw/index.html). The percentage identity

Figure 2

Field population of *Haslea* sp. Typical bluish color of diatoms attached to *Padina* sp. is clearly visible of the 18S rRNA gene fragment of the studied *Haslea* population with other sequences from the GeneBank was calculated using the NCBI BLASTN program, version 2.2.10. Sequences were blasted to confirm the identification (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Morphology

Living cells of *Haslea* sp. showed two narrow band-like, parietal plastids (Fig. 3) lying appressed on each side of the valve margin and not reaching the apex as observed in other species of *Haslea*. Cell tips were characteristically intensely blue coloured. This peculiar bluish pigment accumulating at the cell apices does not occur outside the genus *Haslea*, supporting a close taxonomic relationship within the said genus. Only faint striation was visible in LM (Fig. 3); cells were 41.5 to 90.4 μm long (average 60.1 μm), and 5.8 to 7.8 μm wide (average 7.6 μm) (Table 1).

Diversity of 18S rRNA gene sequences

Our analysis of 18S rRNA gene sequences has revealed that *Haslea* sp. from Saharun is phylogenetically extremely similar to *H. provancialis*, strain RG-2015a in 99.7% and *H. ostrearia* in 97.16%. Further, *Haslea* sp. showed 94.09% similarity with the sequence from the strain *H. spicula* BA28, as well as 94.33 and 94.34% sequence similarity with the strain *H. crucigera* and *H. pseudostrearia* (Table 2), respectively.

Discussion

At present, the genus *Haslea* includes more than 20 species that show significant differences in their morphology, ecology or physiology.

The first molecular phylogeny of some *Haslea* species constructed using chloroplast 16S rDNA (Pillet et al. 2011) supported the division of this genus into several subgenera. However, with the recent discovery of the new species of blue diatoms: *H. karadagensis*,

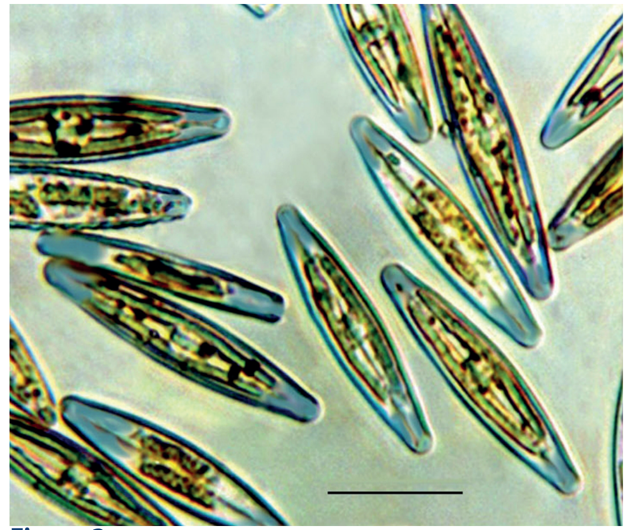

Figure 3

Light microscopy of *Haslea* sp., with parietal chloroplasts and apices filled with blue pigment. **Scale bar = 30 μm**

H. provincialis as well as *Haslea* sp. described here from Croatia, the relative positions of different clusters of species within the genus *Haslea* and the status of the genus deserve further studies.

Haslea ostrearia Gaillon/Bory (Simonsen) was considered the only species with the worldwide distribution, known for its capacity to synthesize a blue pigment – marennine, which accumulates at cell apices (for historical considerations, see Briée 2010). Recently, new species of diatoms with blue apices producing marennine-like pigments, including the one described here, have been discovered, which questions the alleged uniqueness of *H. ostrearia* and unravels the blue diatom biodiversity.

Blue pigmented hasleoid diatoms were previously found on *Padina* sp. in the Gulf of Naples (Funk 1919), on the shores of different Italian islands (Solazzi & Tolomio 1976; Tolomio 1978; Barone 1979), the Tyrrhenian Sea (Tolomio 1984) and the Adriatic (Molisch 1903; Höfler et al. 1956; Solazzi et al. 1972; Tolomio 1976; 1982; 1988; Tolomio & Bullo 2001a,b). In the above cases, however, the identification of diatoms was based only on light microscopy (LM) and the decisive feature was the presence of blue tips. Our

Table 1

Morphological features of *Haslea* sp. (this study) compared with those of *H. ostrearia*, *H. provincialis*, *H. karadagensis*, *H. crucigera* and *H. pseudostrearia* obtained from the literature

	Haslea sp.	*Haslea ostrearia*	*Haslea provincialis*	*Haslea karadagensis*	*Haslea crucigera*	*Haslea pseudostrearia*
Length (μm)	60.1	71.8	65.8	52.5	55.5	95–97
Width (μm)	7.6	7.3	7.4	8.0	8.8	11–121
Color of cell apices	blue	blue	blue	blue-grey	no	no

Table 2

18S rRNA gene fragments sequence similarities (%) among *Haslea* strains

	Haslea sp. (this study)	*Haslea ostrearia*	*Hasle pseudostrearia*	*Haslea crucigera*	*Haslea spicula* BA28
H. ostrearia	97.16				
H. pseudostrearia	94.34	98.06			
H. crucigera	94.33	97.99	97.75		
H. spicula BA28	94.09	98.35	98.06	98.87	
H. sp. RG-2015a	99.70	96.96	93.64	93.62	93.31

study presents the first description of the Adriatic blue *Haslea* sp. based on light microscopy and 18S rRNA gene fragment sequencing.

Our results demonstrated that the possibly new species of *Haslea* sp. is morphologically similar to *H. ostrearia, H. karadagensis* and *H. provincialis* (Table 1, Fig. 3). Pigmented cells of *Haslea* sp. are difficult to distinguish from *H. ostrearia* and *H. provincialis*, but the pigment color distinguishes it from *H. karagadensis*. All three species are also genetically separated.

Morphological data show a lower maximum length of *Haslea* sp. compared to *H. ostrearia* and *H. provincialis* and higher compared to *H. karadagensis*, suggesting that the taxa should be separated. Further, the comparison of molecular data supports the recognition of the new species of diatoms within the same *Haslea* genus, capable of producing blue pigments.

To date, *H. karadagensis* and *H. provincialis* have only been found in their distinct areas of discovery, i.e. *H. karadagensis* in the Black Sea and *H. provincialis* on the French shores of the Mediterranean Sea. Interestingly, the molecular analyses reveal that each area is inhabited by a genetically distinct subpopulation of *H. ostrearia*. One of them occurs in the Atlantic Ocean, the other one in the Kattegat Strait between Sweden and Denmark, and the third one inhabits Coffin Bay in Australia (Gastineau et al. 2013; 2014). Similar distribution patterns have also been documented for *Thalassiosira rotula*, another cosmopolitan diatom (Whittaker et al. 2012). Our results, describing *Haslea* sp. only from the Croatian coast as well as showing differences in the overall sequence similarity and morphology compared to other *Haslea* species inhabiting different geographical areas, support the view that geographically specific distribution may occur among diatoms. Further, we agree with Gastineau et al. (2016) who suggested that *H. provincialis*, and in our opinion possibly also Croatian *Haslea* sp., may have been observed in the Mediterranean Sea already before. They were reported by e.g. Sauvageau (1906) and various Italian authors, but confused with *H. ostrearia*. Unfortunately, due to the lack of preserved specimens or cultures from these studies, no comparison is possible at the moment. It is necessary, however, to reisolate *Haslea*-like, blue-pigmented diatoms from the entire region of the Mediterranean Sea to verify this statement using modern approaches to species identification.

The discovery of this possibly new species of *Haslea* sp. suggests that *Haslea* diversity has been underestimated and needs further detailed studies. This also raises questions about previously published reports and observations of *Haslea* distribution in the Mediterranean Sea whether these organisms really belong to *H. ostrearia*.

With the recent discovery of the new species of blue diatoms, the relative positions of different clusters of species within the genus *Haslea* and the status of the genus deserve further studies. Our study suggests the existence of a new *Haslea* species, however, this assumption has still to be confirmed by reproductive studies, checking the possibility of interbreeding and sequencing further gene fragments.

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Conflict of Interest: Katarzyna Palinska and Waldemar Surosz declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies involving humans or animals performed by any of the authors.

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