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REGULAR PAPER

Haslea nusantara (Bacillariophyceae), a new blue diatom from the Java Sea, Indonesia: morphology, biometry and molecular characterization

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Background and aims – The present study aims to describe a new species of pennate blue diatom from the genus *Haslea*, *H. nusantara* sp. nov., collected from Semak Daun Island, the Seribu Archipelago, in Indonesian marine waters.

Methods – Assessment for species identification was conducted using light microscopy, Scanning Electron Microscopy and molecular techniques. The morphological characteristics of *H. nusantara* have been described, illustrated and compared to other morphologically similar blue *Haslea* taxa, distributed worldwide. Additionally, molecular characterization was achieved by sequencing plastidial and mitochondrial genomes.

Key results – This new species, named *Haslea nusantara*, cannot be discriminated by its morphology (stria density) but it is characterized by its gene sequences (*rbc*L chloroplast gene and *cox*1 mitochondrial gene). Moreover, it differentiates from other blue *Haslea* species by the presence of a thin central bar, which has been previously reported in non-blue species like *H. pseudostrearia*. The complete mitochondrion (36,288 basepairs, bp) and plastid (120,448 bp) genomes of *H. nusantara* were sequenced and the gene arrangements were compared with other diatom genomes. Phylogeny analyses established using *rbc*L indicated that *H. nusantara* is included in the blue *Haslea* cluster and close to a blue *Haslea* sp. found in Canary Islands (*H. silbo* sp. ined.).

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Conclusions – All investigations carried out in this study show that *H. nusantara* is a new blue-pigmented species, which belongs to the blue *Haslea* clade, with an exceptional geographic distribution in the Southern Hemisphere.

Key words – Indonesia, Bacillariophyceae, blue diatoms, *Haslea*, molecular phylogeny, genomic study.

INTRODUCTION

The pennate diatom *Haslea ostrearia* (Gaillon) Simonsen, has long been known as the only blue diatom ever described, and it is also the type species of the genus *Haslea* Simonsen (Simonsen 1974, Poulin et al. 2019). This microalga produces a water-soluble blue pigment, marennine (Lankester 1886), which accumulates at cell apices and is released into the seawater. This pigment is responsible for the greening of oyster's gills in the Atlantic coast of France (Gaillon 1820). This phenomenon gives a significant economic added value to the French oyster industry, since green oysters, with specific flavour and emerald colour, are rarer and more expensive than ordinary oysters. Furthermore, this blue pigment has biological properties like antioxidant (Pouvreau et al. 2008), antibacterial and antiviral (Gastineau et al. 2012a) and allelopathic effect against other diatoms (Pouvreau et al. 2007a, Prasetiya et al. 2016).

For decades, any record worldwide of a blue diatom was assigned to *H. ostrearia*. However, recent works on the blue *Haslea* using scanning electron microscopy (SEM) observation and molecular approaches have enlightened an unsuspected biodiversity of this genus, with two new species, *H. karadagensis* Davidovich et al. (Gastineau et al. 2012b) col-

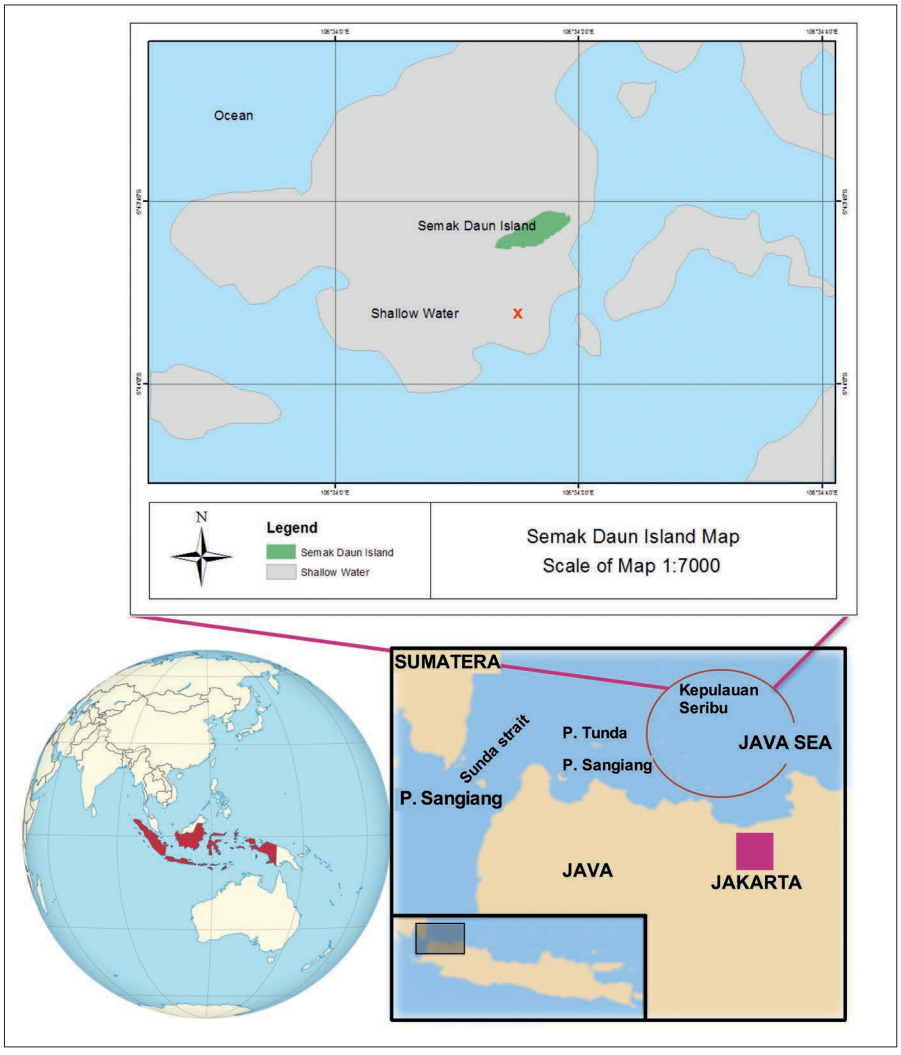

Figure 1 – Sampling location of *Haslea nusantara* near Semak Daun Island (5°43′49.27″S, 106°34′20.27″E), The Thousand Islands ("Kepulauan Seribu"), Indonesia, during spring 2015. Sampling point is indicated with the cross (x) sign. Map produced with QGIS software version 3.6.1 (QGIS Development Team 2018).

lected in the Black Sea, and *H. provincialis* Gastineau et al. (Gastineau et al. 2016) in the Mediterranean Sea. Gastineau et al. (2014) also mentioned one undescribed species yet in the Canary Islands (*Haslea silbo* sp. ined.). Our knowledge about the diversity of non-blue *Haslea* also increased, with *H. sigma* Talgatti et al. found in salt marshes in Southern Brazil (Talgatti et al. 2014), and seven species more from different collections worldwide (Sterrenburg et al. 2015). To date, the genus *Haslea* encompasses 35 taxa as listed in AlgaeBase (Guiry & Guiry 2018) (see http://www.algaebase.org/), but recently, Li et al. (2017) revisited the *Haslea* phylogeny, using SSU rDNA and *rbc*L data, demonstrating that two species must be transferred to the genus *Navicula* Bory, *H. tsukamotoi* Sterrenburg & F.Hinz and *H. avium* Tiffany et al. Most of *Haslea* species have been encountered in the Northern Hemisphere.

The present study aims to describe a new species of pennate blue diatom from the genus *Haslea*, *H. nusantara* sp. nov., collected from Semak Daun Island, the Seribu Archipelago, in Indonesian marine waters. Morphological, biometrical (using SEM) and molecular investigations (chloroplast and mitochondrial genomes) were carried out and spectral analysis of the pigment in the culture supernatant was made. The specificity of this Indonesian new species was established as compared with other known blue *Haslea* species.

MATERIAL AND METHODS

Diatom sampling, isolation and culture

Specimens of *H. nusantara* were sampled using 50 mL conical centrifuge tubes (Thermo Fisher Scientific, France), on 12 Mar. 2015 by scraping the biofilm covering *Padina* sp. thalli growing in the subtidal zone of Semak Daun Island (5°43′49.27″S, 106°34′20.27″E) in the Seribu Archipelago ("Kepulauan Seribu"), Indonesia (fig. 1). A few days after the sampling, six single cells (IND1 to IND6) were isolated from one tube at Le Mans Université (France), using micropipettes and grown in Petri dishes containing modified artificial seawater (Mouget et al. 2009). The *H. nusantara* cultures were grown in Erlenmeyer flasks in a controlled temperature room at 16 ± 1°C and an irradiance of 50–100 µmol photons m^{-2} s^{-1}, provided by fluorescent tubes (Philips TLD 36W/965), during a 14:10 h light:dark cycle. Among the six cells initially isolated, only one (IND6) survived and the strain was maintained in culture for several months.

Microscopic observations

The preparation of samples was performed as described in Kaczmarska et al. (2005). Samples were prepared using a Millipore vacuum filtration apparatus with 25 mm diameter and 3 µm pore size polytetrafluoroethylene (PTFE) membrane (General Electric Osmonics, Minnetonka, USA). Samples were washed with distilled water, re-suspended in 5 mL distilled water and cleaned by adding 10 mL of concentrated sulfuric and nitric acid in a water bath at 100°C for 60 min. Samples were then re-washed using distilled water in the filtration apparatus and re-suspended in 5 mL distilled water. Cleaned samples were then mounted on glass slides using Naphrax and examined using an Olympus BX51 light microscope (LM) with a DP72 digital camera, equipped with phase contrast and differential interference contrast (DIC) optics.

For SEM examination, cleaned diatom frustules and isolated valves were air-dried onto a circular coverslip, mounted on an aluminum stub prior to coating with platinum or palladium-platinum using a JEOL JUC-5000 sputter coater or JEOL JFC-2300 HR high-resolution fine coater, respectively.

Genome sequencing, annotation and analysis

Total DNA was extracted as described in Doyle & Doyle (1990). An Illumina library of 250 base pairs (bp) DNA inserts was prepared and sequenced on the HiSeq 4000 platform by the Beijing Genomic Institute (Shenzhen). A total of 11 million paired-end reads of 150 bp were assembled using Ray version 2.3.0 (Boisvert et al. 2012) and a k-mer value of 45. Contigs of chloroplast and mitochondrial origins were identified by BLAST searches against a local database of organelle genomes and then merged using an overlap-layout consensus approach as implemented in Sequencher version 5.4.1 (Gene Codes Corporation, Ann Arbor, USA). Organelle genomes were annotated using a custom set of tools (Turmel et al. 2015) and circular genome maps were drawn using OGDraw (Lohse et al. 2007). Alignments of chloroplast and mitochondrial genomes from diatom taxa belonging to the Naviculales were carried out using the ProgressiveMauve algorithm of Mauve version 2.3.1 (Darling et al. 2010).

Phylogenetic trees were built, based on *rbc*L chloroplast gene and *cox*1 mitochondrial gene to establish the evolutionary process inside the genus *Haslea*. Our choice focused on *rbc*L (ribulose-bisphosphate carboxylase, large subunit gene) and *cox*1 (cytochrome oxidase subunit I) because they are characterized in many *Haslea* species (sequences available in Genbank™). The multiple alignments were carried out using clustalO (https://www.ebi.ac.uk/Tools/msa/clustalo/). The best evolution model was defined using MEGA7 (Kumar et al. 2016). Maximum likelihood (ML) analysis was generated using RaxML (https://embnet.vital-it.ch/raxml-bb, Stamatakis et al. 2008) and MEGA7 with 1000 bootstrap and maximum parsimony (MP) was performed using PAUP (Swofford 1998) and MEGA7 with 1000 bootstrap too.

Pigment characterization with UV-visible spectrophotometry

The marennine-like pigment produced by *H. nusantara* was characterized on cell-free culture supernatant (syringe-filtered on 0.22 µm, Thermo Fisher Scientific) using the Beer-Lambert law. The spectrum was scanned from 200 to 800 nm, in a 5 cm cell using a spectrophotometer (Perkin Elmer, Lambda 25 scan mode). Marennine-like concentration was estimated using the specific extinction coefficient for the extracellular form of marennine (EMn) at the peak wavelength 677 nm (ε_{677} = 12.13 L g^{-1} cm^{-1}), according to Pouvreau et al. (2007b). Spectral characteristics from *H. nusantara* extracellular marennine-like pigment was compared to EMn as control.

RESULTS

Haslea nusantara Mouget, Gastineau & Syakti, **sp. nov.**

Figs 2 & 3

Type material – Indonesia, Seribu Archipelago ("Kepulauan Seribu"), sandy beach in Semak Daun Island (5°43′49.27″ S, 106°34′20.27″E), associated with *Padina* sp. at depths not exceeding 5 m (holo-: PC, slide PC0576262 and SEM stub PC0576265 with acid cleaned embedded material of strain IND6; the cell representative of the holotype is presented in fig. 3B; iso-: BRM, slide BRM Zu11/18 and SEM stub Qu154-2 with acid cleaned embedded material of strain IND6).

Diagnosis – Cells with two parietal, narrow band-like chloroplasts lying appressed on each side of the valve margin and not reaching the apex. Cell apices characteristically blue coloured. Valves narrow and lanceolate with acute apices. The maximum and minimum length of *H. nusantara* was 91.1 and 54.1 µm, respectively (average, 73.9 ± 1.7 µm, n = 33), while the maximum and minimum width was 8.2 and 5.9 µm (average 6.8 ± 0.1 µm, n = 33). The equation of the fitting curve to describe the relationship between the length to width measurements in *H. nusantara* is:

$y = 0.023\ x + 5.06$, $R^2 = 0.164$, n = 33 (1) (fig. 4)

Axial and central areas are indistinct. Raphe is straight with approximate central endings. Internally, the raphe is bordered on one side by an axial costa, while at center on opposite side, a thin and short central bar can be observed. Striation consists of a transapical pattern (34–37 striae in 10 µm)

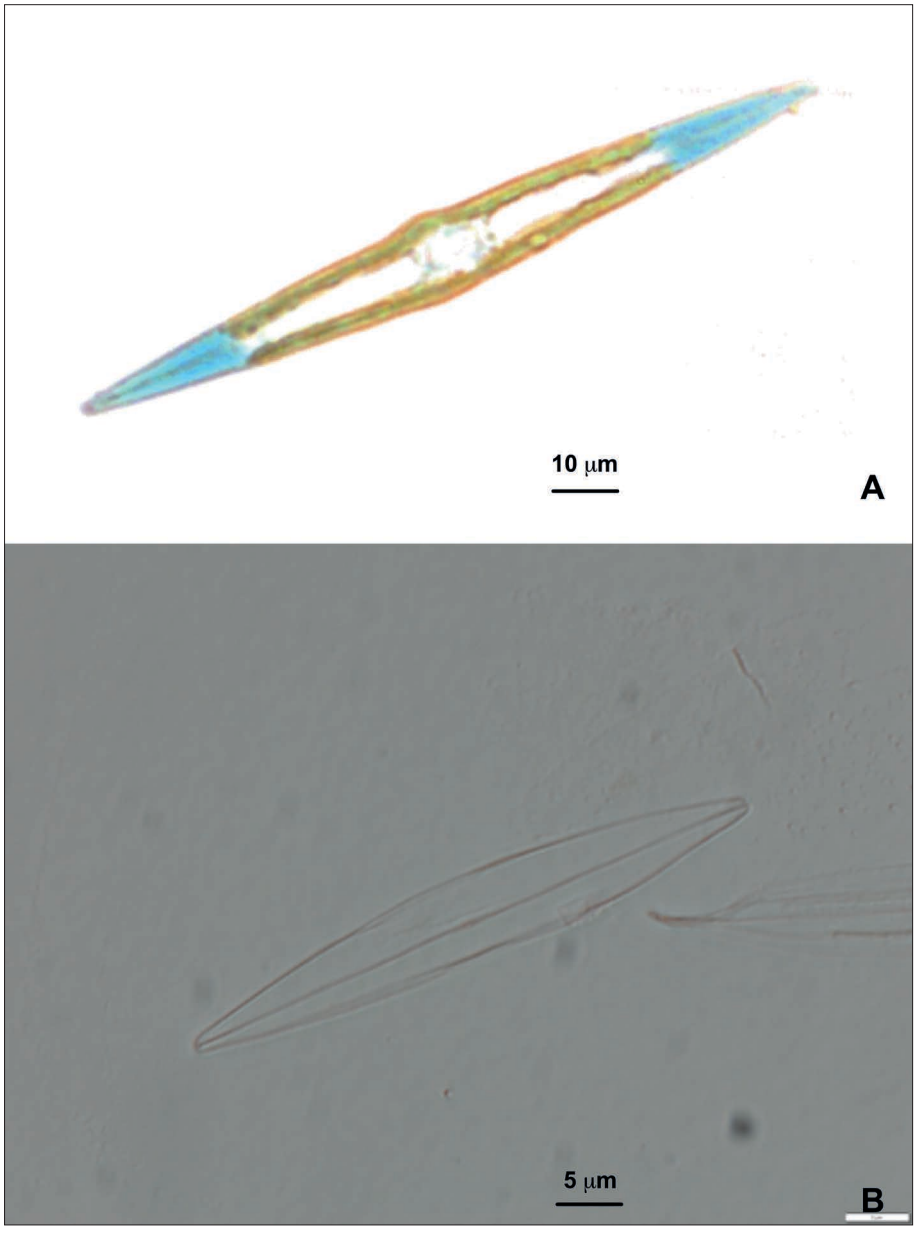

Figure 2 – A, living cell of *Haslea nusantara* with two parietal chloroplasts and apices filled with blue pigment in light microscopy; B, cleaned frustule from the holotype in the Differential Interference Contrast (DIC) mode. Scale bars: A = 10 µm; B = 5 µm.

crossed at right angle by a longitudinal pattern (51–53 striae in 10 µm).

Etymology –The species name refers to the Sanskrit word 'nusantara', which is a contemporary Malay term for the Malay Archipelago and Indonesian Archipelago. The word 'nusantara' itself originated from two Sanskrit words, 'nusa' that means 'island', and 'antara' that means 'in between' or 'including' (Evers 2016). Today, Nusantara covers all Indonesian Archipelago or the national territory of Indonesia, where the species *H. nusantara* has been isolated.

Description – Living cells show the typical coloured areas associated with blue *Haslea* species, which entirely occupy the space immediately beyond the two parietal chloroplasts lying on both valve margins at both apices (fig. 2). Very few details can be seen regarding the valve features in LM (fig. 3). Our SEM observation showed the full view of the internal valve (fig. 3A). The valve appears as narrow and fusiform or lanceolate with acute apices (fig. 3A): 54.1–91.1 µm in length and 5.9–8.2 µm in width. The ornamentation is hardly seen while a median line holding the raphe system runs from one apex to the other (fig. 3A). In SEM, the external valve surface shows distinct linear, fissure-like openings that run continuously throughout the entire length of the valve from one apex to the other (fig. 3A, B & D). Each of these longitudinal fissures merge with a peripheral stria at the valve margin, which both fuse together at the apex beyond the terminal area (fig. 3D). Most of morphological features previously observed on the valve of two other species of blue *Haslea* (Gastineau et al. 2012b, 2016) are present in *H. nusantara*, i.e. lanceolate valves, acute apices and rectangular frustules in girdle view. The true nature of the striation can be best appreciated internally. It consists of a criss-cross pattern with slightly rectangular areolae forming transapical rows that are crossed at right angle by longitudinal rows. There are 34–37 transapical striae in 10 µm (36.0 ± 1.0 in 10 µm, n = 36) and 51–53 longitudinal striae in 10 µm (52.0 ± 0.2 in 10 µm, n = 33). The internal raphe system is composed of two straight branches, which terminate at center in approximate co-axial endings and at the apex in slightly elongated helictoglossa (fig. 3E). There is a narrow and raised up axial costa on one side of the raphe system running almost continuously but stopping shortly before reaching the apices (fig. 3C). In addition, a short (c. 2 µm long) and narrow central bar is present opposite to the axial costa, which is reported for the first time in a blue *Haslea* (fig. 3C).

The comparative analysis of morphological characteristics between *H. nusantara* and other species of the genus *Haslea* described elsewhere (*H. pseudostrearia* Massé et al. and *H. crucigera* (W.Sm.) Simonsen) is presented in table 1. It shows that, biometrically and morphologically, *H. nusan-*

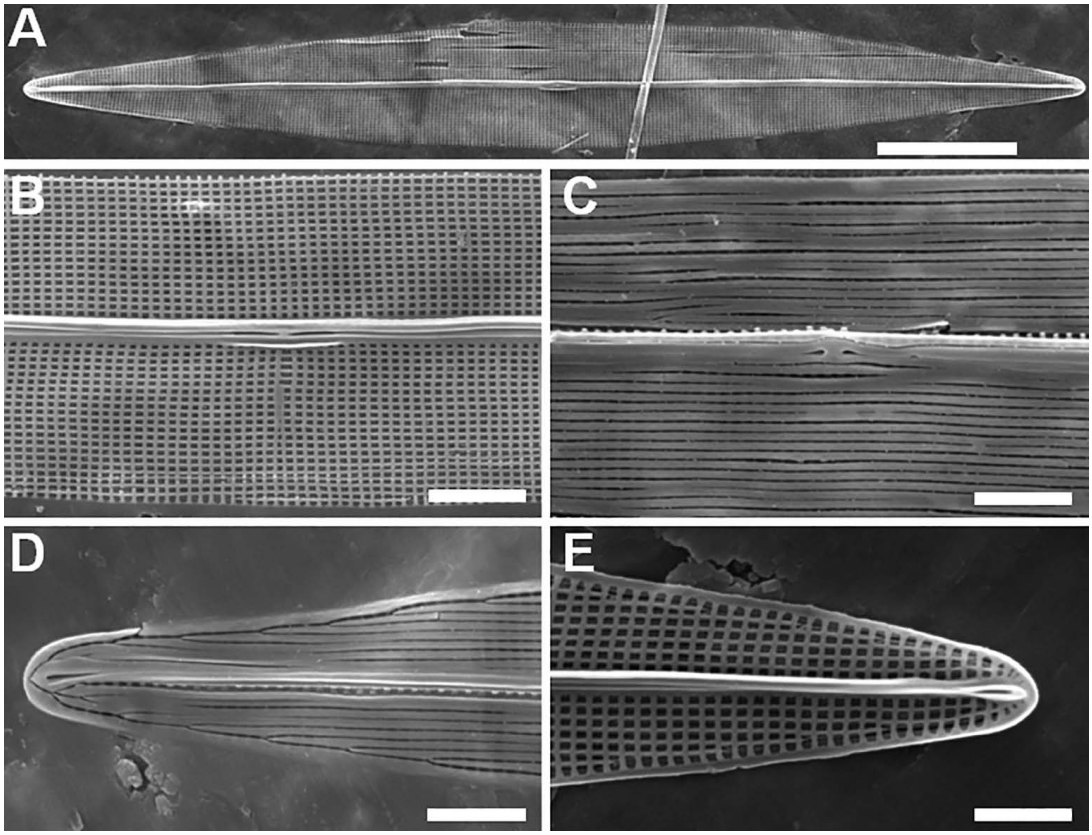

Figure 3 – Holotype material of *Haslea nusantara* from Kepulauan Seribu in scanning electron microscopy: A, whole valve in internal view; B, external view of the valve centre showing approximate raphe endings and continuous longitudinal fissures; C, internal view of the center with criss-cross stria pattern, axial costa on one side of the raphe and central bar in opposite side; D, external view of apex showing the merging of the two peripheral striae beyond the terminal area; E, internal view of apex with the helictoglossa. Scale bars: A = 10 µm; B–E = 2 µm.

Table 1 – **Biometric data and morphological features of *Haslea nusantara* from the present study compared to other *Haslea* taxa from the literature.**
* from Gastineau et al. (2012); ** from Massé et al. (2001); values are given with Standard Error; NA: no data.

	H. nusantara	*H. ostrearia*	*H. provincialis*	*H. karadagensis**	*H. pseudostrearia*	*H. crucigera***
Length (µm)	73.9 ± 1.7 (n = 33)	71.6 ± 5.5 (n = 25)	65.8 ± 0.1 (n = 26)	52.5 ± 0.1 (n = 1148)	55.5 ± 0.2 (n = 25)	95-97
Width (µm)	6.8 ± 0.1 (n = 33)	7.5 ± 1.2 (n = 25)	7.4 ± 0.1 (n = 26)	8.0 ± 0.03 (n = 1148)	8.8 ± 0.1 (n = 25)	11-12
Fitting equation for the length to width ratio	y = 0.02x + 5.06	y = 0.03x + 4.28*	y = 0.02x + 6.33	y = 0.01x + 8.21	NA	NA
Transapical striae in 10 µm	36.0 ± 1.0 (n = 33)	34.8 ± 0.2 (n = 30)	32.7 ± 0.2 (n = 25)	31.4 ± 0.2 (n = 30)	38.6 ± 0.2 (n = 25)	17
Longitudinal striae in 10 µm	52.0 ± 2.0 (n = 33)	53.3 ± 0.2 (n = 30)	60.2 ± 0.2 (n = 25)	57.8 ± 0.3 (n = 30)	42.8 ± 0.2 (n = 25)	20
Pseudostauros	Not present	Not present	Not present	Not present	Not present	Present
Axial costa	Present	Present	Present	Present	Present	Present
Central bar	Present	Not present	Not present	Not present	Present	Present with pseudostauros
Central raphe endings	Straight	Straight	Straight	Straight	Straight	Deflected
Polar raphe endings	Straight	Straight	Straight	Straight	Straight	Deflected
Colour of pigment at apex	Blue	Blue	Blue	Blue-grey	No pigment	No pigment

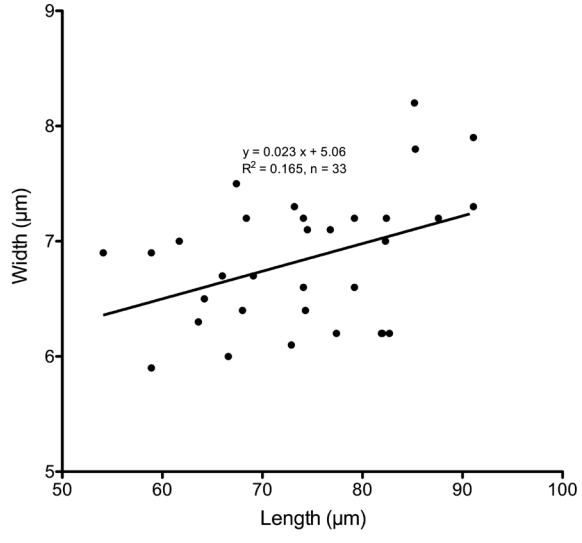

Figure 4 – Distribution of lengths and widths in *Haslea nusantara* from a culture maintained in the laboratory (n = 33). Relationship between mean values of width and length was measured and the obtained equation of the fitting line was: y = 0.023 x + 5.06 (n = 33, R^2 = 0.165).

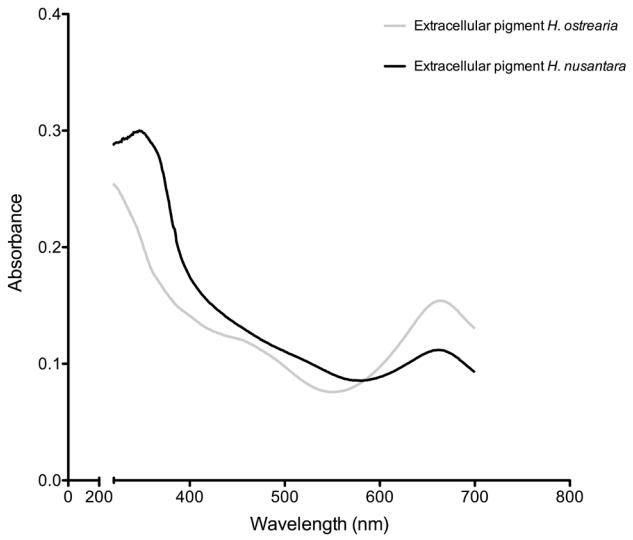

Figure 5 – Absorbance spectrum of *Haslea nusantara* extracellular pigment from 250 to 800 nm at pH 7, compared to that of *H. ostrearia*.

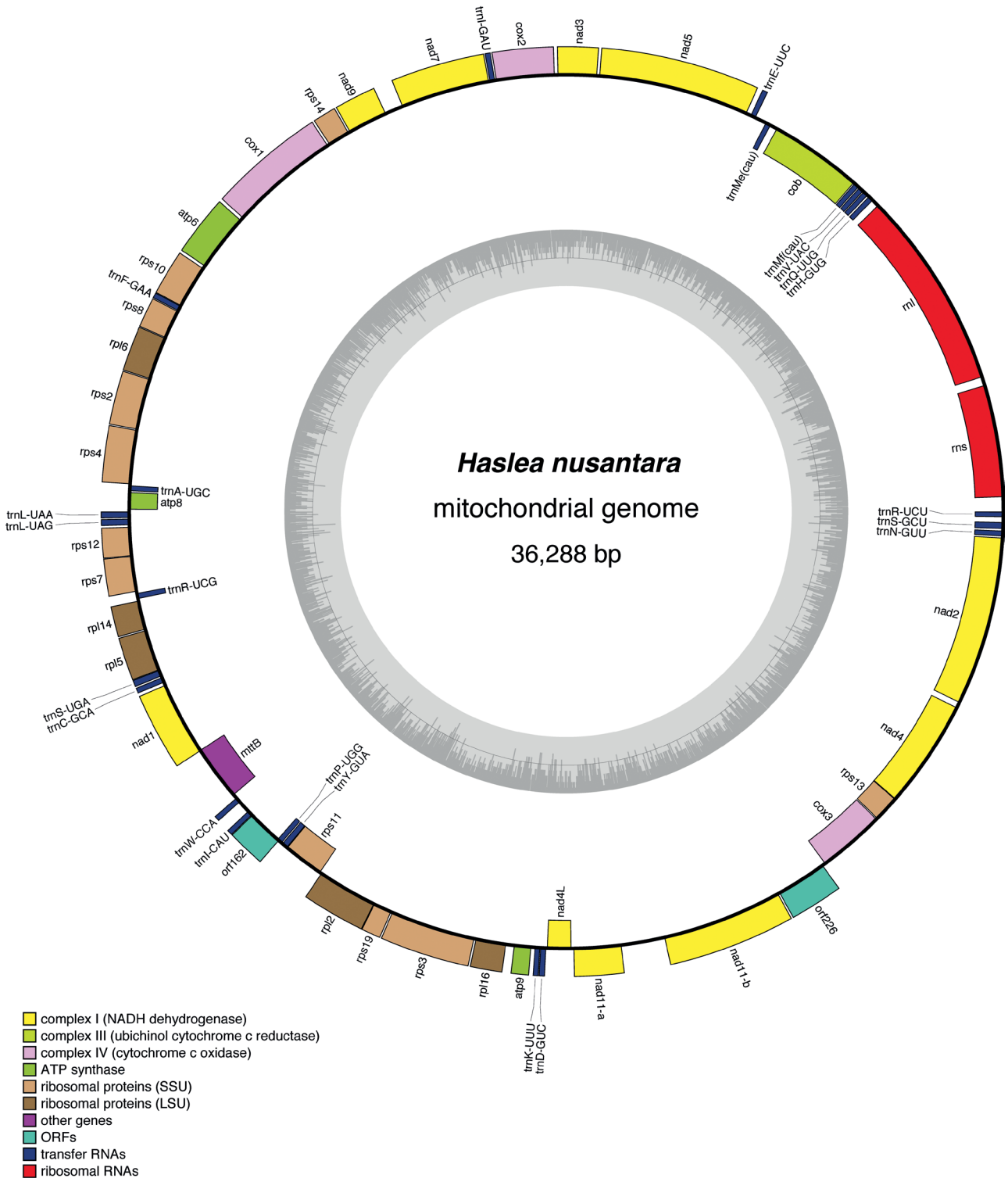

Figure 6 – Gene map of the *Haslea nusantara* mitochondrial genome. Filled boxes represent genes, with colours denoting gene categories as indicated in the bottom left legend. Locally collinear blocks of genome sequences are represented by boxes of identical colour and similarly coloured blocks are connected by lines. Blocks lying above the centre line of the aligned regions are in the same orientation as in the reference (first) genome sequence, while those below this line are in the reverse orientation. Locally collinear blocks of genome sequences are represented by boxes of identical colour and similarly coloured blocks are connected by lines. Blocks lying above the centre line of the aligned regions are in the same orientation as in the reference (first) genome sequence, while those below this line are in the reverse orientation. Genes on the outside of the map are transcribed counter-clockwise; those on the inside are transcribed clockwise. The inner ring shows variations in G+C content with the circle inside the G+C content graph marking the 50% threshold (dark grey, G+C; light grey, A+T). bp: base pair.

Table 2 – **General features of diatom mitochondrial genomes.**

Order	Species	Accession number	Length of base pairs	GC content (%)	Total genes	Protein-coding genes	tRNA gene	rRNA gene	Reference
Fragilariales	*Asterionella formosa*	KY021079	61,877	26.62	62	35	25	2	Villain et al. 2017
Fragilariales	*Ulnaria acus*	GU002153	46,657	31.77	59	33	24	2	Ravin et al. 2010
Thalassiosirales	*Skeletonema marinoi*	KT874463	38,515	29.73	62	35	25	2	An et al. 2015
Thalassiosirales	*Thalassiosira pseudonana*	NC_007405	43,827	30.11	64	35	27	2	Oudot-Le Secq & Green 2011
Bacillariales	*Pseudo-nitzschia*	KR149143	46,283	31.04	62	37	23	2	Yuan et al. 2016
Naviculales	*Fistulifera solaris*	NC_027978	39,476	28.13	63	33	25	2	Tang & Bi 2015
Naviculales	*Halamphora coffeaeformis*	NC_037727	44,653	32.91	44	38	22	2	Pogoda et al. 2018
Naviculales	*Berkeleya fennica*	KM886611	35,509	29.72	63	36	25	2	An et al. 2014
Naviculales	*Phaeodactylum tricornutum*	NC_016739	77,356	35.01	61	34	24	2	Oudot-Le Secq & Green 2011
Naviculales	***Haslea nusantara***	MH681882	36,288	29.24	61	36	22	2	This study

Table 3 – **General features of diatom chloroplast genomes.**

Order	Species	Accession number	Length of base pairs	GC content (%)	Inverse Region (IR)	Protein-coding genes	tRNA gene	rRNA gene	Reference
Fragilariales	*Asterionella formosa*	KC509519	121,238	30.67	Yes	132	31	4	Ruck et al. 2014
Fragilariales	*Ulnaria acus*	JQ088178	116,251	30.56	Yes	129	30	4	Galachyants et al. 2012
Thalassiosirales	*Thalassiosira pseudonana*	EF067921	128,814	30.66	Yes	127	27	4	Oudot-Le Secq et al. 2007
Bacillariales	*Pseudo-nitzschia*	KR709240	111,539	31.37	Yes	96	27	3	Cao et al. 2016
Naviculales	*Fistulifera* sp.	AP011960	134,918	32.20	Yes	132	27	3	Tanaka et al. 2011
Naviculales	*Phaeodactylum tricornutum*	EF067920	117,369	32.56	Yes	130	27	3	Oudot-Le Secq et al. 2007
Naviculales	***Haslea nusantara***	MH681881	120,448	31.10	Yes	134	30	4	This study

tara resembles *H. ostrearia*, for instance regarding the mean length and width of the cell, as well as the number of striae in both transapical and longitudinal axis.

Marennine-like pigment

Measurements using UV-visible spectrophotometry revealed that the extracellular form of *H. nusantara* blue pigment demonstrated spectral characteristics similar to marennine produced by *H. ostrearia*, and to marennine-like pigments produced by other blue *Haslea* species, with two peaks, one in the UV and the other in the red part of the spectrum. The maximum spectral absorbance for *H. nusantara* and *H. ostrearia* at pH 7 in the visible part of the spectra was observed at 663 and 666 nm, respectively (fig. 5). It is very similar to the extracellular form of marennine (EMn) produced by *H. ostrearia*.

Structure and organization of organelle genomes

We have sequenced both the mitochondrial and chloroplast genomes of *H. nusantara* and compared them with those of previously examined diatoms. The *H. nusantara* mitochondrial genome was assembled as a circular DNA molecule of 36,288 bp that encodes 36 proteins, 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs) (table 2 & fig. 6). The accession number of this mitogenome is MH681882. It is among the smallest diatom mitochondrial genomes that have been completely sequenced to date but is in the same size range as other naviculalean genomes (Pogoda et al. 2018). At 29.24%, its G+C content is similar to those of several other diatom mitochondrial genomes (Pogoda et al. 2018). Comparative analyses of gene order using MAUVE revealed that the *H. nusantara* and *Berkeleya fennica* Juhlin-Dannfelt mitochondrial genomes are colinear, except for a single sequence inversion (fig. 7). In contrast, the gene ar-

rangement of *H. nusantara* is highly scrambled with respect to those of all other naviculaleans.

The 120,448-bp chloroplast genome (accession number: MH681881) of *H. nusantara* features the typical quadripartite architecture observed in many algae (Turmel & Lemieux 2018, Yu et al. 2018); two copies of a large inverted repeat (IR) sequence of 7,241 bp divide the *H. nusantara* genome into a large (63,119 bp) and a small (42,847 bp) single-copy regions (table 3 & fig. 8). The circular chloroplast genome encodes 132 proteins, 27 tRNAs and three rRNAs, with the IR including the *ycf89*, *psbY* and *trnP*(ugg) genes in addition to the rRNA operon (fig. 8). Currently available diatom chloroplast genomes all feature the quadripartite architecture (Yu et al. 2018) and tend to be more conserved in size than their mitochondrial counterparts (tables 2 & 3). MAUVE alignments of the chloroplast genomes of *H. nusantara* and two other naviculaleans showed that the gene order of *H. nusantara* more closely resembles that of *Phaeodactylum tricornutum* Bohlin than that of *Fistulifera solaris* Mayama et al. (fig. 9).

Phylogenetic analysis

A phylogenetic analysis was carried out to establish the relationship between *H. nusantara* with other *Haslea* species. The best model selected by MEGA7 to *rbc*L fragment (1336 bp) was GTR+G+I (BIC: 7055.021, AIC: 6751.877, lnL: -3336.850, +G parameter: 0.5359, +I: 60.2 % evolutionarily invariable sites). The base frequencies calculated were respectively: f(A) = 0.296, f(T) = 0.321, f(C) = 0.177 and f(G) = 0.206. The phylogenies (ML/MP), generated using RaxML (ML), PAUP (MP) and MEGA7 (ML/MP) (to compare the topologies generated) showed that *H. nusantara* is included in the blue-pigmented *Haslea* species clade (robust node: 99/92), and it may be sister species of *H. silbo* sp. ined. (node: 60/73) (fig. 10).

On the other hand, phylogenetic analysis (ML, MP) carried out using RaxML, PAUP and MEGA7 (to compare the results) on mitochondrial *cox*1 fragment (696 bp), was based on best model HKY+G (BIC: 6089.902, AIC: 5942.063, lnL: -2948.949, +G parameter: 0.3229). The base frequencies were f(A) = 0.252, f(T) = 0.411, f(C) = 0.162 and f(G) = 0.176,

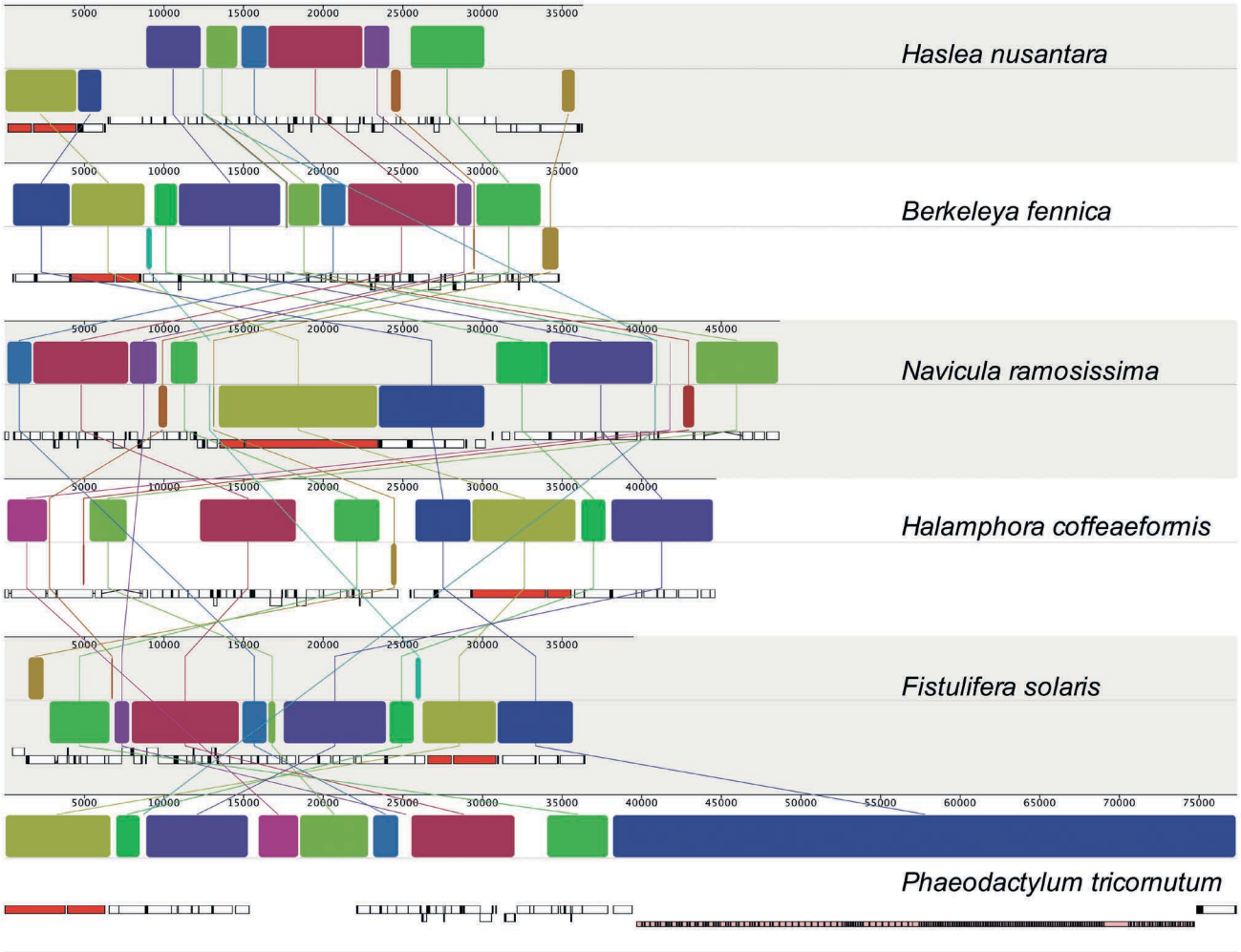

Figure 7 – Extent of mitochondrial genome rearrangements in naviculalean diatoms. The genome alignments were carried out using the Progressive Mauve algorithm of Mauve version 2.3.1 (Darling et al. 2010).

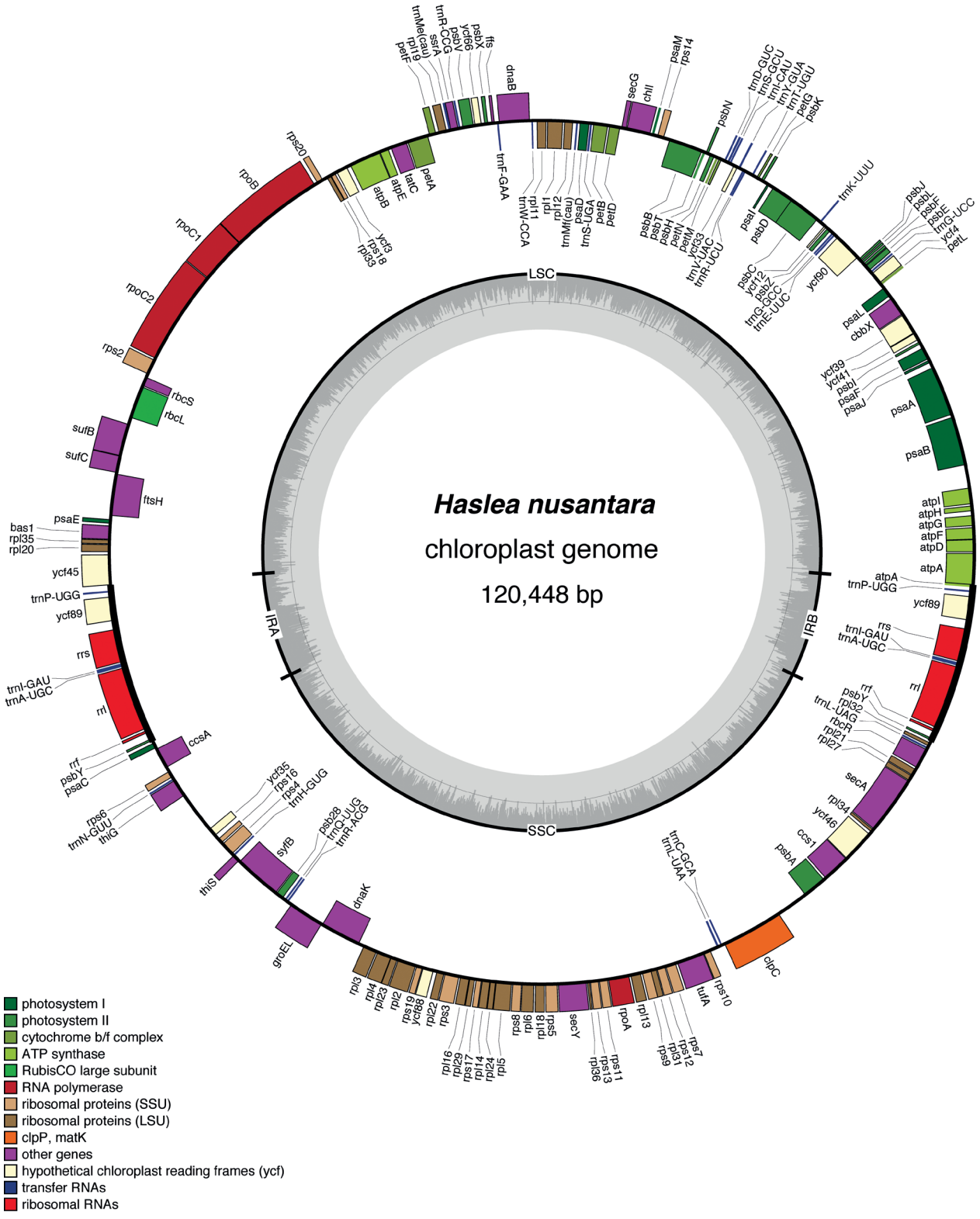

Figure 8 – Gene map of the *Haslea nusantara* chloroplast genome. Filled boxes represent genes, with colours denoting gene categories as indicated in the bottom left legend. Locally collinear blocks of genome sequences are represented by boxes of identical colour and similarly coloured blocks are connected by lines. Blocks lying above the center line of the aligned regions are in the same orientation as in the reference (first) genome sequence, while those below this line are in the reverse orientation. Genes on the outside of the map are transcribed counter-clockwise; those on the inside are transcribed clockwise. The inner ring shows variations in G+C content and the positions of the IR and single-copy regions (SSC and LSC). The circle inside the G+C content graph marks the 50% threshold (dark grey, G+C; light grey, A+T). bp: base pair.

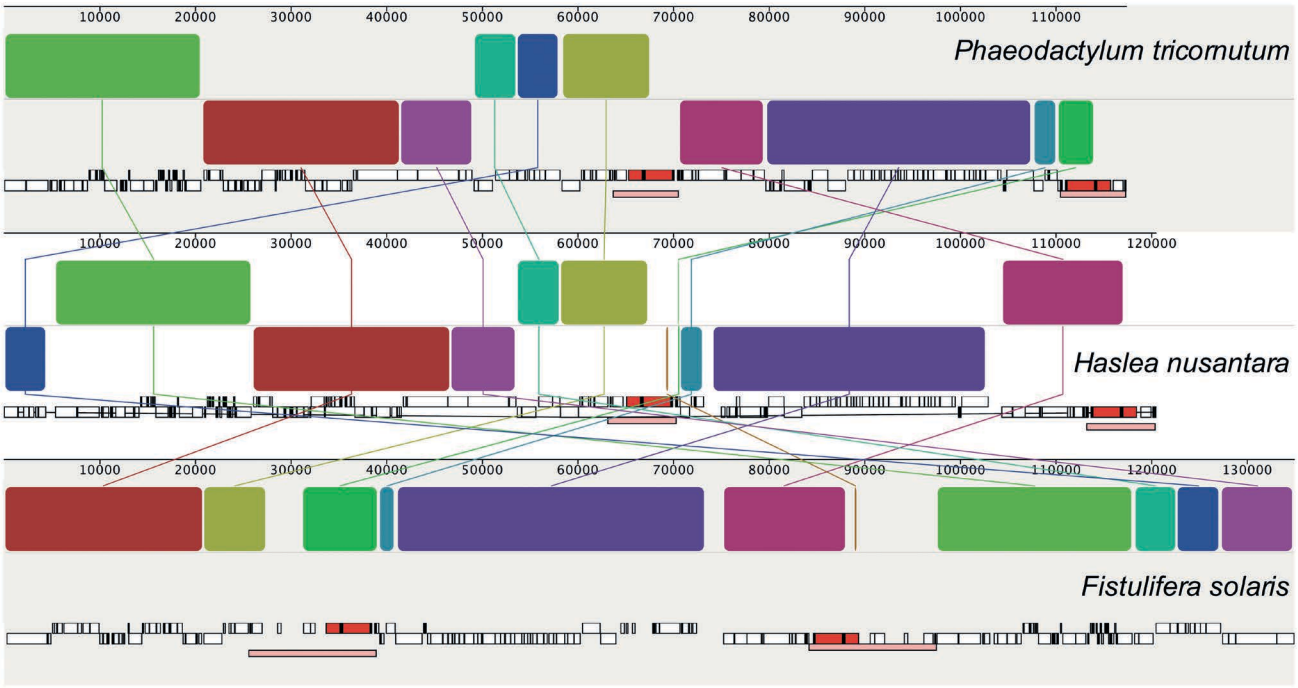

Figure 9 – Extent of chloroplast genome rearrangements in three naviculalean diatoms. The genome alignments were carried out using the Progressive Mauve algorithm of Mauve version 2.3.1 (Darling et al. 2010).

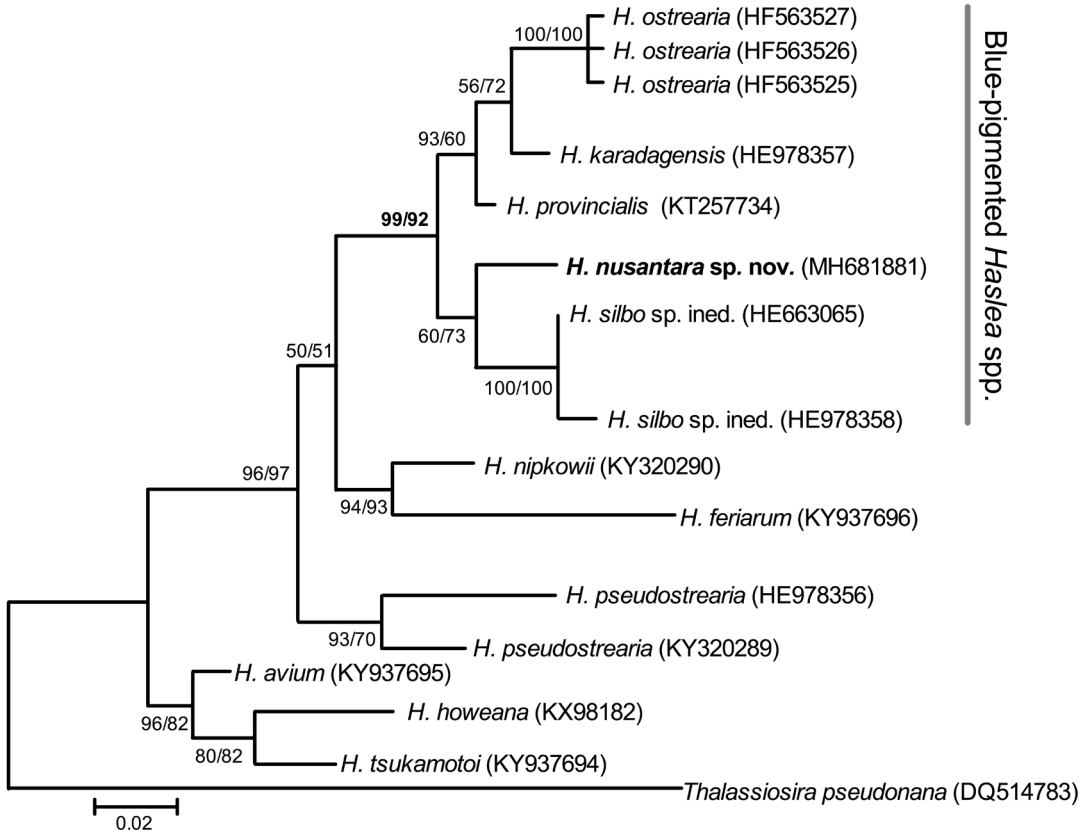

Figure 10 – Maximum likelihood (ML) phylogenetic tree based on *rbc*L (chloroplast gene) from 11 *Haslea* species. The bootstrap values are given for ML and maximum parsimony (ML/MP) based on 1000 replicates.

respectively. The topologies obtained were similar whatever the software used, showing a minor resolution for relationship between the blue *Haslea*, when compared to *rbc*L analysis. *Haslea nusantara* and *H. ostrearia* seem genetically sdissimilar. There is strong support (node: 98/100) for a close relationship between *H. provincialis* and *H. silbo* sp. ined. (fig. 11). In both, *rbc*L (fig. 10) and *cox*1 (fig. 11) trees, *H. nusantara* lies on long branches suggesting that it has diverged considerably from other blue-pigmented *Haslea* species.

DISCUSSION

Considered as one of the largest archipelagos in the tropical region, Indonesia is one of the marine biodiversity hotspots (Roberts et al. 2006). Although recently some works described Indonesian diatom flora, most of them only concentrated on freshwater species (Bramburger et al. 2008, 2017, Bramburger & Hamilton 2014) and only a few on marine environment (Sterrenburg et al. 1995, Hendrarto & Nitisuparjo 2011). The diversity of marine diatoms from Indonesia thus remains underexplored. Furthermore, up to now, most of the blue *Haslea* species have been mainly discovered in the Northern Hemisphere, with the exception of the type species *H. ostrearia*, with specimens observed in the Indian Ocean (Simonsen 1974), but for these are neither SEM pictures nor genetic data available, as for populations of *H. ostrearia* living in the Australian waters (Gastineau et al. 2014). The discovery of a blue *Haslea* species in the Java Sea is thus a novelty.

Characteristics of the new taxon *Haslea nusantara*

Cells of *H. nusantara* were collected in biofilms associated to *Padina* thalli, and when in culture, they formed aggregates and biofilm on the bottom of the flasks. In light microscopy, as for other blue *Haslea*, *H. nusantara* cells appeared lanceolate and free living, with apices filled with a typical blue pigment. The blue colour in *H. nusantara* was similar to those of *H. ostrearia* and *H. provincialis* but different from that of *H. karadagensis*, which is more blue-greyish (Gastineau et al. 2012b). The UV-visible spectrophotometry revealed that at pH 7, the extracellular form of the pigment produced by *H. nusantara* displays a maximum absorbance in the red region with little difference when compared to marennine (663 vs. 666 nm, respectively). The peak of maximum absorbance in the red region measured in this study for marennine is comparable to those observed in previous works (Pouvreau et al. 2006a, 2006b, 2007b). The spectra being pH-dependent, in absence of more material, however, it cannot be assessed if the differences between pigments of both *Haslea* species are significant. Furthermore, the *H. nusantara* strain having been lost before running further experiments, we were not able to characterize its intracellular form. Therefore, we could not compare this measurement with the intracellular form of marennine (IMn) from *H. ostrearia*.

Observation of cleaned frustules using light microscope revealed that the striation in *H. nusantara* is almost invisible, confirming that this method is of limited use for *Haslea* species identification. In SEM, however, *H. nusantara* is

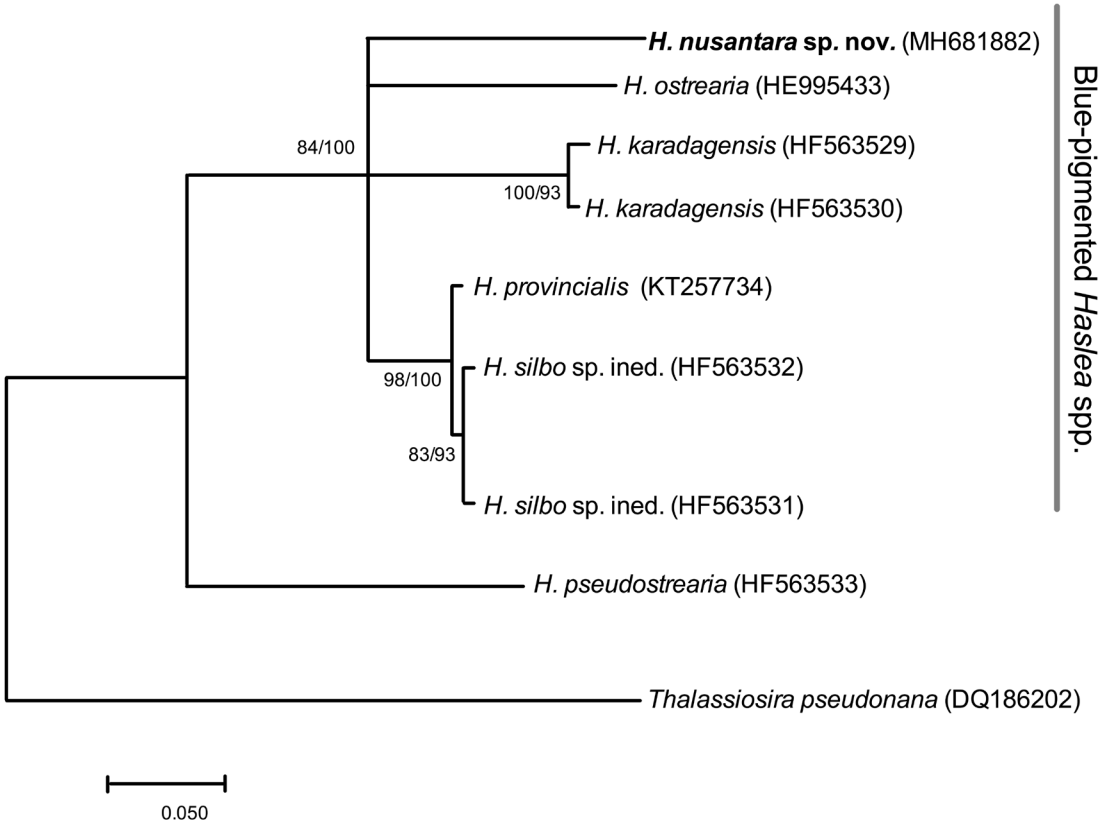

Figure 11 – Maximum likelihood (ML) phylogenetic tree based on *cox*1 (mitochondrial gene) from six *Haslea* species. The bootstrap values are given for ML and maximum parsimony (ML/MP) based on 1000 replicates.

biometrically and morphologically similar to the previously described blue *Haslea* species, for instance *H. ostrearia*, *H. karadagensis* and *H. provincialis*, with lanceolate valves, acute apices in valve view and rectangular frustules in girdle view (Gastineau et al. 2012b, 2016). If the fitting equation for the length to width ratio in *H. nusantara* cells differs from the ones in *H. ostrearia*, *H. karadagensis* and *H. provincialis* (table 1), the stria density is very similar for both *H. nusantara* and *H. ostrearia*. From these results, it can be inferred that biometrically *H. nusantara* is very similar to *H. ostrearia*, however, using molecular markers, there is no doubt that *H. nusantara* is a new species of blue *Haslea*. Indeed, according to the genetic markers sequenced and the phylogenetic trees, *H. nusantara* is included in the blue-pigmented *Haslea* spp. cluster, and this new species seems distinct from *H. ostrearia* and possibly close to *H. silbo* sp. ined.

The Indonesian blue diatom inside the genus *Haslea*

A global consideration of the 35 species of *Haslea* identified so far allows distinguishing different features regarding their morphology, ecology and physiology. For instance, *H. gigantea* Simonsen has been identified as a mesoplanktonic species with a cell length that may reach 500 µm. In contrast to most *Haslea* species that are free benthic organisms, *H. crucigera* (W.Sm.) Simonsen is known to be a tube-dwelling species, and *H. wawrikae* (Hust.) Simonsen is planktonic. The diversity in the frustule morphology can also be viewed within *Haslea* species. For example, a pseudostauros (thickening of the transapical central virgae into thick ribs) is present in *H. crucigera*, *H. crucigeroides* (Hust.) Simonsen and var. *densestriata* Cardinal et al., *H. salstonica* Massé et al. and *H. spicula* (Hickie) Bukht. (Cardinal et al. 1984, Massé et al. 2001). A molecular phylogeny approach using 16S rDNA in several species suggested the division of the genus *Haslea* into sub-genera, discriminating between taxa with a pseudostauros (*H. crucigera* and *H. salstonica*) on the one hand, the sigmoid *H. nipkowii* (F.Meister) Poulin & Massé (Poulin et al. 2004), the non-blue *H. pseudostrearia* and the blue *H. ostrearia* that do not have a pseudostauros on the other hand, and a third group composed of the planktonic *H. wawrikae* (Hust.) Simonsen (1974) (Pillet et al. 2011). From the present work, it can be assessed that the new species *H. nusantara* refers to benthic pennate diatoms, with a marennine-like pigment accumulating at cell apices, and a frustule devoid of a pseudostauros but with a short and narrow central bar at the valve centre.

Lastly, the new species *H. nusantara* has been discovered in a geographical region different from the previous ones (*H. karadagensis*, *H. provincialis* and *H. ostrearia*), which all were from the Northern Hemisphere. This suggests that the diversity of this peculiar taxon is undoubtedly underestimated, and that there are probably more species of blue *Haslea* to discover.

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