Sexual reproduction in the newly–described blue diatom, *Haslea karadagensis*

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Abstract: For decades, the diatom genus *Haslea* has been known to comprise both "colorless" species and one species containing a blue pigment, the latter being known as *H. ostrearia*. Recently, a new blue diatom named *H. karadagensis* has been isolated from the Black Sea. The mating compatibility of the two species has been tested, and their reproductive isolation confirmed. We provide a detailed description of the heterothallic sexual reproduction process in this new species. Cells from clones which are sexually compatible arrange gametangial pairs. Each gametangium in the pair produces two gametes, which to a large extent are morphologically and behaviorally isogamous. No mucilage or mucilage structures are observed. Zygotes and auxospores have no contact with parental frustules, and their orientation is rather irregular though they tend to lie parallel to each other. Evaluation of the position of cardinal points in the life cycle of the species, and the rate of cell size decrease in culture are presented. In the breeding system of this species both homo– and heterothallic ways of reproduction are realized. The latter is basic and predominant. Among the 36 clones investigated, 16 were sexually compatible with the other 20. Homothallic behavior was extremely rare; only one clone displayed a homothallic mode of reproduction.

Key words: auxosporulation, *Haslea karadagensis*, heterothallism, sexual reproduction

Introduction

The diatom genus *Haslea* Simonsen is well known partly because of *Haslea ostrearia* (Gaillon) R. Simonsen, which produces the specific pigment "marennine" and is responsible for the greening of oysters. Several occurrences of *H. ostrearia* have been reported worldwide, without any serious doubts being raised about the identification of this species. Recently, a pennate diatom with colored apices resembling *H. ostrearia* was isolated from the Black Sea. Some morphological features of the two species turned out to be very similar, linking them to the same genus *Haslea*. However, examination of a full range of characteristics, including the morphology of the frustules and pigment features (optical characteristics in UV spectrophotometry and the Raman spectral signature) allowed us to differentiate *H. ostrearia* from this newly isolated diatom, which has been named *H. karadagensis* (Gastineau et al., submitted). Diatom classification, which was long based mainly on morphological features, has now adopted new methods, with molecular genetic analysis being the most modern (Medlin 2003; Mann & Evans 2007). Preliminary investigations indicated molecular differences between *H. ostrearia* and the closest relative, *H. karadagensis*. Unfortunately, the methods of molecular analysis, even though they are sensitive, objective, and applicable even at the subspecies level, are not indicative of a species difference *per se* (e.g. Orsini et al. 2002; Cerino et al. 2005). They do not prove that evolutionary divergence has been completed, considering emerging reproductive isolation. In some cases, it is virtually impossible

to assess solely from the results of genetic analysis whether plainly–distinguishable groups of clones represent several morphotypes/genotypes of the same species or separate species (e.g. KACZMARSKA et al. 2009). In such cases, the concept of the biological species, which is based on the ability to interbreed, is a likely approach to be applied (MANN 2010), and is increasingly being used (AMATO et al. 2007; VANORMELINGEN et al. 2007, 2008; CASTELEYN et al. 2008; D'ALELIO et al. 2009; KACZMARSKA et al. 2009; TROBAJO et al. 2009).

Sexual reproduction of *H. ostrearia* was first observed in monoclonal cultures (NEUVILLE & DASTE 1975, 1979), where it was homothallic, as these authors did not attempt to cross–breed their strains. The heterothallic mode of reproduction was not induced and reported until three decades later (DAVIDOVICH et al. 2009), and a sexual process corresponding to type IB2a according to GEITLER's (1932, 1935) system was ascertained. In the present paper we describe and illustrate the sexual reproduction of *H. karadagensis*, the species from the genus *Haslea* isolated from the Black Sea, and demonstrate its sexual incompatibility with *H. ostrearia*. The cardinal points in the life cycles of the two species are compared, and their breeding systems analyzed.

Materials and methods

Over a period of three years (2008–2011) a total of 41 clones of *H. karadagensis* were derived from natural population that inhabits the pebble sublittoral near the Karadag biological station, Ukraine (44° 54' 41'' N, 35° 12' 04'' E). Samples were collected at a depth of 0.2–0.4 m. Monoclonal cultures were started as single cells isolated by micropipettes, and were designated as Y.MMDD–Z, where Y is the last number of the year of isolation, MM is the month, DD is the date, and Z is a short form of the name of the clone. Clonal and mixed cultures used for testing sexual compatibility were incubated in glass Petri dishes (50–60 mm diameter) in artificial seawater, ESAW medium (ANDERSEN et al. 2005, p. 494). Mixtures of clones were produced using exponentially–growing cultures. Natural lighting from a north–facing window at 20±2 °C, or in low light under 'cool–white' fluorescent tubes (*c*. 20 μmol photons.m^{-2}.s^{-1}), and with a long–day artificial photoperiod, 14/10 h light/dark cycles, was used to ensure better vegetative growth, and the best sexual productivity was achieved at lower irradiances (<50 μmol photons.m^{-2}.s^{-1}) and with shorter photoperiods (6–10 h) (unpublished results). During a period of one week the mixed cultures were inspected daily for signs of auxosporulation under an MBS–9 light microscope (LOMO, Leningrad, Russia), or a Nikon TS100 inverted microscope, using bright field (BF) optics. Sexual reproduction was deduced from the formation of gametes, zygotes, auxospores or initial cells. Photoimages were captured by Canon PowerShot A95 or Canon PowerShort A640 digital cameras through a Biolar PI microscope (PZO, Warsaw, Poland), equipped with a water immersion objective x40, numerical aperture 0.65, operating in differential interference contrast (DIC) mode. The cell sizes were measured with a precision of 1.72 μm by using an ocular–ruler calibrated against an object–micrometer. Apart from the cells grown in the laboratory, the lengths of 1149 cells obtained from a natural population between 09 April and 08 May, 2008 were measured. Data for the cell sizes of *H. ostrearia* were obtained from the clones studied previously (DAVIDOVICH et al. 2009), their sexual descendants, and some new clones obtained from samples collected in the oyster ponds of C. Pénisson (46° 59' 19'' N; 2° 14' 14'' W). Mating incomparability of two species, *H. karadagensis* and *H. ostrearia*, was checked in mixtures of clones which were sexually competent and readily enter sexual reproduction if mixed inside the species. Before mating experiments, all the clones were acclimated during a week to the same salinity level of 30‰. The influence of salinity on vegetative growth rate and reproductive frequency was checked in the range of salinity from 8 to 40‰. Cultures maintained at 30‰ were acclimated to a chosen level of salinity (17, 23, 30, 40‰) during a week. Two–step acclimation was applied to reach lower levels, initially one week at 17 and then one week at 8 or 12‰. Every day during four days after reinoculation, the number of cells was counted in 20 fields of microscope view directly in Petri dishes, by using a water immersion objective. The growth rate (division per day) was calculated according to the exponential model. Reproduction frequency was calculated as a relative number of generative cells to the total number of generative and vegetative cells. We regarded cells as generative if they took part or arose in the process of sexual reproduction, i.e. gametangia, gametes, zygotes, auxospores, and initial cells. Two gametes were counted as a single generative cell. Mean values are presented as mean ± standard error, referring *n* to the number of measurements.

Results

Life form and life cycle

Haslea karadagensis was comparatively abundant in the samples collected in the vicinity of the Karadag Biology Station during the spring of years 2008, 2009, and 2010. The life form of the species is that of solitary highly motile cells, which are easily distinguishable in the samples

Table 1. Summary of the cell length ranges in two *Haslea* species.

	Haslea ostrearia			*Haslea karadagensis*		
	min	max	*n* [a)]	min	max	*n*
Vegetative cells	16	143	1484	22	97	1593
Gametangia	16	68	160	22	52	80
Initial cells	75	143	249	64	97	138

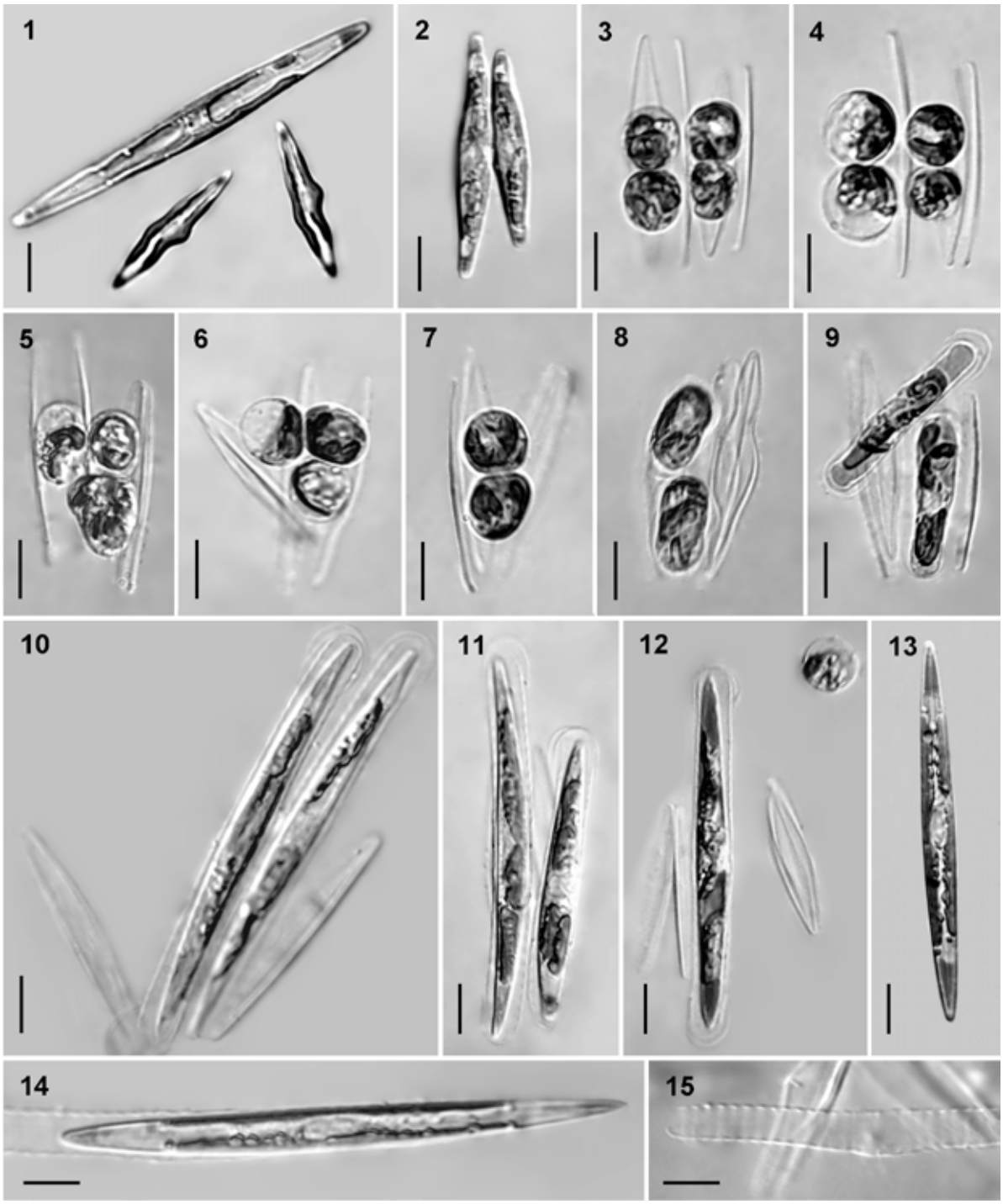
[a)] *n* = number of cells measured; min and max are minimal and maximal apical cell sizes occurred, μm.

even under low magnification, because of their characteristic lancet shape and blue tips, with two big chloroplasts per cell, lying either side of the girdle and occupying around 2/3 of the cell length (Fig. 1). A central cytoplasmic bridge across the vacuole contains the nucleus. The length of cells in the field population varied from 29 to 84 μm. Based on these data, and on measurements of cells used in our experiments, the full size range of the species *Haslea karadagensis* can be evaluated to span from 97 to 22 μm (Table 1). Cells became sexually inducible after their size decreased below 52 μm, and they maintained an ability to reproduce sexually down to the smallest cell sizes observed (22 μm). The sexually–inducible size range occupies 40% of the full size range. Initial cells resulting from sexual reproduction ranged in size from 64 to 97 μm, thus covering 44% of the total species–specific size range.

Cell sizes were measured at intervals of two to seventeen months in thirteen clones maintained in culture under natural light from north window, and at a temperature of 19–21°C. This allowed us to calculate a mean cell size reduction rate, which was equal to 2.85±0.42 μm/month (*n*=13).

Sexual reproduction

Sexual reproduction in the species investigated in heterothallic pairs of clones began by the pairing of two cells (gametangia) originated from different clones. Heterothallism was easily inferred from the different cell sizes of the clones (Figs 2–4). The gametangia positioned themselves girdle–to–girdle by active gliding (Fig. 2). Pairing involved no visible (under BF or DIC optics) accumulation of mucilage, such as occurs in some other raphid diatoms. When the cells were paired, the chloroplasts changed position, moving from the girdle zone to the valves (compare Fig. 1 and Fig. 2), but did not divide. At the end of meiosis, each gametangium contained two spherical gametes, lying either side of the median transapical section (Fig. 3). As this occurred, the gametangial thecae were usually forced apart. Gamete development in one gametangium occurred more or less synchronously with that in the other gametangium, but not perfectly. It could be often seen that the gametes in one gametangium appeared to be slightly swollen (Fig. 4). Syngamy took place between the gametes closest to each other: first one pair of gametes fused and then, after an interval of a few minutes to tens of minutes, the next pair fused (Figs 5, 6). During meiosis, each gamete inherited one of the two chloroplasts of the parental cell, and as a result of plasmogamy, the zygote contained two plastids transmitted bi–parentally (Fig. 5). The zygotes tended to be formed between the gametangial thecae, and within a short time after syngamy each zygote contracted a little and, as a result, was approximately the same size as a single gamete (Fig. 7). There was no close contact between zygotes and gametangial thecae, and there was no visible mucilage capsule around them. The disposition of the gametes was therefore rather irregular relative to the gametangial frustules. Within an hour, the zygotes started to swell, and from this time they can be designated as auxospores (Fig. 8). Expansion was bipolar, and was not strictly oriented relative to gametangial thecae (Fig. 9); there was only slight tendency of auxospores to expand parallel to each other, and more or less parallel to the parental frustules (Figs 10–12). The growing auxospores contained a blue pigment that was clearly visible in the space free of chloroplasts (Fig. 9), and in the zygotes and gametes. Expanding (Fig. 9) and fully–developed (Fig. 10) auxospores retained the remnants of a zygote envelope as caps at the cell ends. Normally each gametangial pair produced two initial cells of similar size (Fig. 10), but sometimes the initial cells in a pair were noticeably different in size



Figs 1–15. *Haslea karadagensis* vegetative and generative cells, light microscopy, differential interference contrast: (1) during the life cycle the cell apical length decreases up to four fold, cells growing in culture may get deformities; (2) pairing of gametangia is the first visible sign of the sexual reproduction process, note different size of cells belonging to two sexually compatible clones; (3) each gametangium in a pair produced two spherical gametes; (4) sometimes gametes of one gametangium are slightly swelled; (5) the moment of gametic syngamy (lower pair of gametes); (6) two gametes just before fusion (above) and a young zygote (below); (7) two resulted zygotes are surrounded with gametangial thecaes but have no tight contact with them; (8) zygotes started to grow, from this moment they may be termed auxospores; (9) zygote envelope remains are seeing as "caps" at the ends of growing auxospores, note cytoplasm at the ends of auxospores colored with marennine; (10) initial cells formed inside the fully developed auxospores; (11) the auxospore extension process is not fully synchronized, in a pair one auxospore may stop to grow earlier and form shorter initial cell; (12) just one zygote in a gametangial pair developed into initial cell, while another one aborted at the early stage; (13) tips of initial cells are typically colored with marennine; (14) escape of the initial cell from the perizonium envelope; (15) perizonium has visible transverse bands. Scale bar 10 μm.

Table 2. Results of crossing of *Haslea karadagensis* clones.

Clone name	8.0407-S	8.0408-A	8.0410-D	8.0417-A	8.0417-C	9.0522-H	9.0522-I	9.0522-R	9.0526-D	9.0602-B	0.0511-C	0.0511-F	0.0511-H	0.0511-N	0.0511-R	0.0511-Z	1.0607-C	1.0607-H	1.0607-M	8.0408-B	8.0417-B	8.0417-D	8.0424-A	8.0424-C	8.0424-E	8.0424-G	8.0508-B	8.0508-C	(8.0424-G)-intra-8.0929-A	9.0522-A	9.0522-D	9.0522-X	9.0825-A	9.0825-M	0.0511-A	0.0511-B	0.0511-J	0.0511-M	0.0511-P	0.0511-Q	1.0607-A	1.0607-N	Mating type
	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	
8.0407-S																																											b
8.0408-A	0																																										b
8.0410-D	0	0																																									b
8.0417-A	0	0	0																																								b
8.0417-C	.	0	.	.																																							b
9.0522-H	.	.	.	.	.																																						b
9.0522-I	.	0	.	.	0	.																																					b
9.0522-R	.	.	.	.	.	.	.																																				b
9.0526-D	.	.	.	.	.	.	.	.																																			b
9.0602-B	.	.	.	.	.	.	.	.	.																																		b
0.0511-C	.	.	.	.	.	.	.	.	.	.																																	b
0.0511-F	.	.	.	.	.	.	.	.	.	.	0																																b
0.0511-H	.	.	.	.	.	.	.	.	.	.	0	.																																b
0.0511-N	.	.	.	.	.	.	.	.	.	.	0	.	.																																b
0.0511-R	.	.	.	.	.	.	.	.	.	.	0	.	.	.																																b
0.0511-Z	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.																															b
1.0607-C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.																														b
1.0607-H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.																													b
1.0607-M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.																												b
8.0408-B	+	1	+	+	2	.	3	.	.	.	.	.	.	.	.	.	.	.	.																										r
8.0417-B	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0																										r
8.0417-D	2	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.																										r
8.0424-A	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.																									r
8.0424-C	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	0	.																									r
8.0424-E	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.																								r
8.0424-G	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	[+]																							r
8.0508-B	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.																					r
8.0508-C	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.																				r
(8.0424-G)-intra-8.0929-A	.	2	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.																		r
9.0522-A	.	.	.	.	2	.	.	3	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0																r
9.0522-D	.	.	.	.	.	2	2	2	2	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.															r
9.0522-X	.	2	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	0	0														r
9.0825-A	.	.	.	.	.	.	3	.	.	.	2	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.														r
9.0825-M	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.													r
0.0511-A	.	.	.	.	.	.	.	.	.	.	3	2	2	3	3	2	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.												r
0.0511-B	.	.	.	.	.	.	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.											r
0.0511-J	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.										r
0.0511-M	.	.	.	.	.	.	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.									r
0.0511-P	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.								r
0.0511-Q	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.							r
1.0607-A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.						r
1.0607-N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	2	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.					r

Note. Relative abundance of the sexual reproduction was conventionally ranged: (3) abundant; (2) not rare; (1) rare; (0) absence; (+) reproduction was recorded without ranging; (.) mating was not fulfilled; ([+]) intraclonal reproduction; intra- = clone resulted from intraclonal reproduction; dash outline indicates a zone of mating compatibility; (b) mating type «blue»; (r) mating type «red».

Table 3. Results of crossing of *Haslea karadagensis* and *H .ostrearia* clones.

			Haslea ostrearia								*Haslea karadagensis*													
	Clone name		I7	Sauv3	S133	H3	HSV13	I2	I5	I6	8.0407-S	8.0417-A	9.0522-I	9.0526-D	9.0602-B	0.0511-C	0.0511-H	8.0408-B	8.0424-A	8.0424-C	8.0424-G	8.0825-M	9.0522-A	0.0511-A	Location
Haslea ostrearia	I7	(=NCC158.4)*									0	0	0	0	0	0	0	0	0	0	0	0	0	0	Atlantic Ocean, France
	Sauv3	(=NCC234.1)	0								0	0	0	0	0	.	.	0	0	0	0	0	0	.	Atlantic Ocean, France
	S133	(=NCC148.73)	0	0							0	0	.	.	.	.	.	0	0	0	0	0	.	.	Atlantic Ocean, France
	H3	(=NCC157.3)	+	+	+						0	0	0	0	0	.	.	0	0	0	0	0	0	.	Atlantic Ocean, France
	HSV13	(= NCC321)	+	.	.	.					.	.	.	.	.	0	0	.	.	.	.	.	.	0	Atlantic Ocean, Sweden
	I2	(= NCC158.1)	+	+	+	0	.				0	0	.	.	.	.	.	0	0	0	0	0	.	.	Atlantic Ocean, France
	I5	(= NCC158.2)	+	+	+	0	.	0			0	0	.	.	.	.	.	0	0	0	0	0	.	.	Atlantic Ocean, France
	I6	(= NCC158.3)	+	+	+	0	.	0	0		0	0	.	.	.	.	.	0	0	0	0	0	.	.	Atlantic Ocean, France
Haslea karadagensis	8.0407-S																								Black Sea, Karadag
	8.0417-A										0														Black Sea, Karadag
	9.0522-I										.	.													Black Sea, Karadag
	9.0526-D										.	.	0												Black Sea, Karadag
	9.0602-B										.	.	0	0											Black Sea, Karadag
	0.0511-C										.	.	.	.	.										Black Sea, Karadag
	0.0511-H										.	.	.	.	.	0									Black Sea, Karadag
	8.0408-B										+	+	.	.	.	.	.								Black Sea, Karadag
	8.0424-A										+	+	.	.	.	.	.	0							Black Sea, Karadag
	8.0424-C										+	+	.	.	.	.	.	0	0						Black Sea, Karadag
	8.0424-G										+	+	.	.	.	.	.	0	0	0					Black Sea, Karadag
	8.0825-M										+	+	.	.	.	.	.	0	0	0	0				Black Sea, Karadag
	9.0522-A										.	.	+	+	+	.	.	.	.	.	.	.			Black Sea, Karadag
	0.0511-A										.	.	.	.	.	+	+	.	.	.	.	.	.		Black Sea, Karadag

Note. Sexual reproduction was detected (1) or not detected (0) in the mixture; (.) mating was not fulfilled; dash line outlines a sector of the sexually compatible partners; (*) corresponding names given to the clones in Nantes Culture Collection are shown in brackets.

(Fig. 11). In some cases only one initial cell of a pair survived (Fig. 12). The perizonium was well developed, with conspicuous transverse bands (Figs 10–12, 14–15), 4.2 ± 0.1 in 10 μm (n=26, range 3.3 – 5.1). All transverse perizonial bands, with the exception of the central or primary band, had approximately the same diameter. The auxospore stopped expanding when 13–17 transverse bands had been produced on either side of the primary band. Because of the lancet shape of the initial cell laid down inside the perizonium with parallel walls, there was a noticeable gap between the wall of perizonium and thecae of the initial cells, which broadened toward the end of the cell (Figs 10–12). Some of the initial cells were slightly bent (Figs 10, 11); others were not (Figs 12, 13). The initial cells escaped from the perizonium by active gliding (Fig. 14). The tips of the initial cell were normally colored by blue pigment (Fig. 13).

Breeding system

Sexual reproduction was achieved mainly in the mixtures of clones which were sexually compatible (Table 2), thus demonstrating that heterothallic reproduction did occur. We could not observe intraclonal reproduction directly, and so we cannot describe the pattern of homothallic sexual reproduction, although there was indirect evidence that it happened once in clone 8.0424–G, where initial cells resulting from sexual reproduction were found. Isolated homothallic descendant (8.0424–G)–intra–8.0929–A was of the same sex as the parent clone, and was shown to be sexually compatible with clones of the opposite sex. Several auxospores were found in the mixture of clones of the same sex, 8.0408–B and 8.0424–C, which we attributed to homothallic reproduction of one or both clones. No other occurrences of homothallic reproduction were recorded. If clones were sexually compatible and had suitable cell sizes, gametes and young zygotes were usually seen 3–5 days after exponentially growing clonal cultures had been mixed. If two sexually–compatible clones had differing cell sizes, the members of each of the gametangial pairs in the mixture of these clones were unequal in length, which suggested a heterothallic mode of reproduction.

Numerous attempts to mate *Haslea ostrearia* from the Atlantic Ocean with *H. karadagensis* from the Black Sea were unsuccessful (Table 3). We used different clones, including control pairs of each species that reproduced vigorously when mated separately. Over two years more than 20 interbreeding experiments were carried out with the clones of both species grown at the same culture conditions, favorable for reproduction, i.e. salinity 30‰, low irradiance, and short photoperiod (Mouget et al. 2009). In every experiment, sexual reproduction was observed in the pairs of clones consisting of the same species, but not in mixed pairs. We did not check the breeding compatibility of the species producing a blue pigment with strictly different colorless members of the genus *Haslea.*

Relation to salinity

H. karadagensis was capable to grow at different salinities in the range from 8 to at least 40‰ (Fig 17). Cells were alive at 8 ‰ but their growth rate was close to zero. Shift of salinity from lower to higher levels was more favorable for vegetative growth if compared with the opposite change from higher to lower levels. Salinity of 25–30‰ was optimal for vegetative growth. Unlike vegetative growth, sexual reproduction was impossible at salinity less than 17‰ (Fig. 18). Higher levels of salinity were favorable for sexual reproduction. Moreover, the more shift of the salinity level was applied in the range from 17 to 40‰, the more frequency of reproduction observed in clones acclimated to 17, 23, and 30‰. The highest reproduction frequency was achieved if clones acclimated to 17‰ were transferred (reinoculated) into 40 ‰ medium.

Discussion

Mating experiments allowed us to demonstrate reproductive isolation between Atlantic *H. ostrearia* and *H. karadagensis* from the Black Sea. These two species are essentially different from other members of the genus; because of the specific blue pigments they produce. Their explicit inability to interbreed is evidence in favor of the Biological Species Concept, and confirms the existence of "biological species" in diatoms in particular (see Mann 1999, 2010). Morphological, physiological, biochemical, and genetic differences are useful, and can very often be used to differentiate between species, but they are not sufficient if we are trying to find out whether a particular population has divided during the course of its evolutionary history or not. In this respect

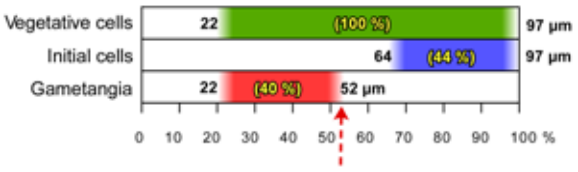


Fig. 16. Diagram showing the spans of the life cycle stages in *Haslea karadagensis*. The percentage of the full size occupied by a particular stage is shown in brackets. The upper size limit critical for sexualization is indicated by a dotted arrow.

inherited in generations, or from the fact that one gametangium had accidentally released its gametes before the other. An unfavorable disposition of the gametangial frustules might further impede immediate copulation, thus leading to swelling of the gametes released first. Gamete behavior of this type could formally be classified as anisogamous (*cis*–anisogamy), which corresponds to category IA2 in Geitler's classification (GEITLER 1973; ROUND et al. 1990; MANN 1993). However, from the data obtained we cannot conclude that this differentiation in behavior corresponds to the two sexes. In many other cases, the gametes appear to be both morphologically and behaviorally similar, and the pattern of sexual reproduction in these cases can be assigned to the Geitler's type IB2a. Isogamous behavior has also been shown to be typical of *H. ostrearia* (DAVIDOVICH et al. 2009).

H. karadagensis has a well–developed perizonium like *H. ostrearia* (DAVIDOVICH et al. 2009). In both species the initial valves do not exactly match the internal shape of the perizonium. The lancet form of the initial cells differs significantly from the tube shape of the perizonium. This implies that the perizonium does not simply act as a "mold" for the initial cell, and the regulation of the process of shaping the initial cell is obviously more complex. Slight curvature is also a characteristic of initial cells (Figs 10, 11, 14), which is not unusual (COX 2010), as are the not–unusual cell deformities (Fig. 1) acquired by cultured specimens during their life history (KOCIOLEK & STOERMER 2010).

The mating system of these species combines two modes: homothallic and heterothallic. However, while heterothallism was an almost constant feature of the 37 clones studied when they were mated in various pair–wise combinations, homothallic reproduction was detected in only one monoclonal culture. Furthermore, heterothallic reproduction was much more productive, resulting in tens to hundreds of gametangial pairs per Petri

we should acknowledge that, despite certain similarities between their morphological features, such as pigment characteristics *etc.*, Atlantic *H. ostrearia* and Black Sea *H. karadagensis* are distinct biological species, and they are both different from other *Haslea* species.

Twofold difference in the level of salinity of water in Atlantic Ocean and the Black Sea cannot be regarded as a factor that prevents interbreeding. Literature data (WRAIGE et al. 1998; DAVIDOVICH et al. 2009) and our experiments show that these two species are osmotolerant organisms which have relatively broad and overlapping salinity tolerance ranges; they grew and reproduced sexually most copiously at 25–30‰. Moreover, increase of salinity was favorable for sexual reproduction of *H. karadagensis* and correlation between the increment of salinity and frequency of sexual reproduction was positive (Fig. 18).

The life cycle of *H. karadagensis* can be regarded as typical of many other pennate diatoms. Cells become sexually inducible after their apical length has reached a cardinal point (*sensu* GEITLER 1932) of around 52 µm. This critical threshold corresponds to 54% of the maximum species–specific size in this species (Fig. 16), and fits in with the general tendency in diatoms (DAVIDOVICH 2001). The sexually–inducible size is open–ended. This means that even the smallest cells at the end of their life span can still engage in sexual reproduction. Smaller gametangia usually give rise to smaller initial cells, while bigger gametangia produce bigger initial cells (DAVIDOVICH 2001). In our case, the size restitution factor found for the smallest gametangia was equal to 64/22=2.9, whereas that for the biggest ones was 97/52=1.9. Taking into account a cell size reduction rate of 2.85 µ.month^{-1}, the total duration of the life cycle can be calculated to be (97–22)/2.85=26 months or 2.2 years for the biggest initial cells, and markedly shorter for the smallest ones, (64–22)/2.85=15 months or 1.2 year. The biggest initial cells take about (97–52)/2.85=16 months to reach the sexually–inducible size range, whereas the smallest initial cells take only (64–52)/2.85=4 months. These are a very rough estimation, and correspond to the growth conditions used here. Moreover, we know nothing about the change in the cell size reduction rate during the life cycle, something that has been observed, for example, in *Pseudo–nitzschia* species (AMATO et al. 2005; D'ALELIO et al. 2009). A life cycle lasting more than one year (2–40 y) is thought to be common

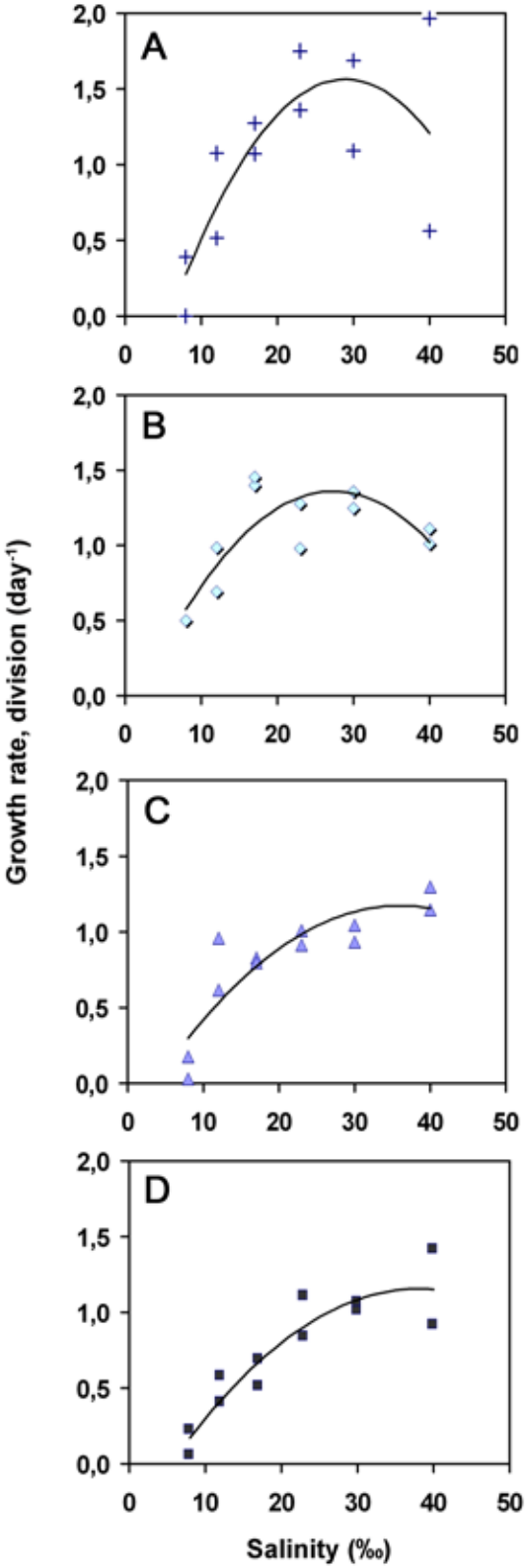


Fig. 17. Dependence of the growth rate of *Haslea karadagensis* on the salinity level. Before experiment cultures were acclimated during a week to salinities 12 (A), 17 (B), 23 (C), and 40 ‰ (D). Data are smoothed by quadratic polynomial.

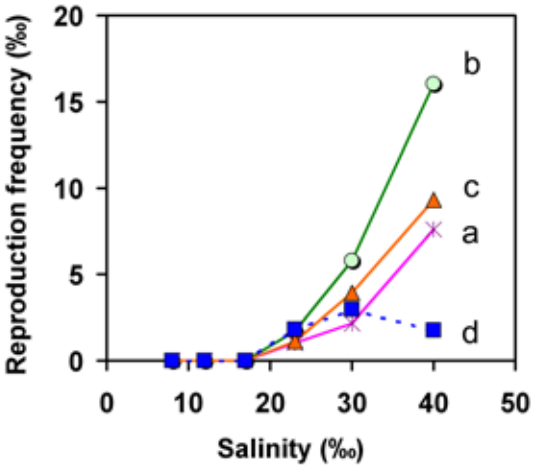


Fig. 18. The effect of salinity on the frequency of sexual reproduction. Each point corresponds to a mean value obtained as a result of mating of two pairs of sexually compatible clones 0.0511–A + 0.0511–N and 0.0511–C + 0.0511–M. Before mating clones were acclimated during a week to salinities 12 (a), 17 (b), 23 (c), and 40 ‰ (d).

among diatoms (Lewis 1984; Mann 1988; Jewson 1992; D'Alelio et al. 2010). Our calculations suggest that sexual events may occur annually in culture; however we cannot say anything about the natural population of *H. karadagensis*, given that there is no information on how fast cells divide in natural conditions.

In *H. karadagensis*, the gametangial apical size region occupies 40% of the total size range in this species, which is very similar to the situation in *H. ostrearia* (41%). The initial cell size region amounts to 44 and 54% of the total size in *H. karadagensis* and *H. ostrearia,* respectively. In some diatoms the smallest initial cells resulting from sexual reproduction are small enough to be sexualized, and thus able to embark on a new round of auxosporulation immediately (Roschin 1994; Chepurnov et al. 2004). Multistep auxosporulation and size restitution have not previously been recorded in *H. karadagensis* or *H. ostrearia*, and theoretically, in view of the absence of overlap of the size regions of initial and gametangial cells, it seems unlikely to occur.

There is some uncertainty about the pattern of sexual reproduction in the species investigated. In some gametangial pair we could see that both gametes in one gametangium were slightly swollen (Fig. 4). This may promote syngamy, but might be also a single way to achieve contact between gametes, as no one form of gamete motion was observed. Slight asynchrony could have resulted either from anisogamy related to the sexes and

dish (bottom area *c.* 20 cm^2), in contrast to just a few occurrences of auxosporulation in the case of intraclonal reproduction. Unfortunately, the environmental cues that trigger homothallic reproduction in some *H. karadagensis* strains are still unknown, as it is in *H. ostrearia* strains.

Depending on their mating compatibility, the clones could be divided into two groups, mating types of which we designated conventionally as "red" and "blue". The mating type capable of intraclonal reproduction was classified as "red". A total of 19 clones from a natural population were found to be type "blue", and 22 were "red". The ratio of sexes in the population could be regarded thus as more or less equal.

In conclusion, it should be emphasized that species description and taxonomy that in diatoms have so far been based predominantly on morphological features (COX 2009, 2010; MANN 2010) need to be accompanied by another approaches. The reproductive biology methods can provide most decisive characteristics (MANN 2010). To reach a comprehensive description of a species, its reproductive biology characteristics must be regarded not as a "good–looking" supplementation but as an integral part of the species diagnosis. Life cycle, sexual behavior, and breading system are the most important characteristics of the species biology. From this standpoint, the results reported here represent the "end point" in the description of *H. karadagensis*. Furthermore, the data obtained are also important for analyzing the discrepancies/similarities between representatives of the genus *Haslea*. So far, the reproductive biology of two species of the genus, namely *H. ostrearia* and *H. karadagensis,* has been described. The investigation of sexual reproduction patterns of colorless, sternum–bearing members of the genus appears to be prospective.

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