Sexual Reproduction of the Black Sea Diatom

Climaconeis scalaris (Brébisson) E.J. Cox

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Abstract—The paper reports a process of sexual reproduction in the Black Sea diatom Climaconeis scalaris (Brébisson) E.J. Cox, 1982, initiated in laboratory conditions. A crossing system of this species allows both homo- and heterothallic reproduction. The fertilization is allogamous. Diploid mother gametangial cells secreted mucilage, forming a mucilage track, while actively gliding relative to each other. Each gametangium in a pair produced two elongated, cylindrical round-ended haploid gametes, which were largely morphologically and behaviorally isogamous. The division of the protoplast of the gametangial cell occurred in the transapical plane without the rearrangement of gametes. Growing auxospores were located parallel to the parent frustules. The research produced data on changes in the cell size, number of chloroplasts, and the position of cardinal points in the life cycle of the examined species.

Keywords: diatoms, Climaconeis scalaris, clonal cultures, vegetative growth, sexual reproduction, auxosporulation.

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Taxonomy of the genus Climaconeis Grunow, 1862, had for long remained debatable. There are 18 species known and described in this genus at present [1]. A significant contribution to its taxonomy has been made by Cox [2, 3], Prasad [4], and Lobban et al. [5]. Cox examined the history of nomenclature and taxonomy of the genus in 1982. As a result of an extensive and thorough analysis of the literature and primary materials, she detected a close resemblance between Okedenia Eulenstein ex G.B. De Toni, 1891, gen. and Climaconeis gen. and proposed to combine them into the one genus, specifically, Climaconeis, which has a nomenclatural priority over Okedenia [3]. The majority of Climaconeis spp. are tropical with only three, that is, C. delicatula (Cleve) E.J. Cox, C. inflexa (Brébisson ex Kützing) E.J. Cox, and C. scalaris, found in moderate latitudes [6, 7]. Frustules of Climaconeis can be curved or straight; the majority of species are easily recognized in the type material under a light microscope by the presence and position of H-shaped chloroplasts with a central pyrenoid [2, 4].

The group of raphid pennates to which Climaconeis gen. belongs is the largest among the main phylogenetic lineages of diatom algae and comprises the majority of the current species and genera. Notwithstanding, 99% have not been described in terms of their interbreeding systems and process of sexual reproduction. The latter has not been previously studied in any Climaconeis spp. The article reports data with respect to sexual reproduction of C. scalaris from the Black Sea for the first time.

MATERIALS AND METHODS

Natural populations of C. scalaris inhabit the sublittoral zone near Karadag biological station. Individual cells, which gave rise to clonal cultures, were isolated using micropipettes from samples in the form of scrapes of fouling from stones taken from the 20–50 cm depth in the proximity to Kuzmichev’s Stone (44°54’41” N, 35°12’45” E). The cultures were maintained in glass Petri dish and Erlenmeyer flasks on modified medium ESAW [8] in natural light coming through a north-facing window at a constant temperature of 20 ± 2°C. In the beginning, the clonal cultures were kept in the medium of salinity at 18‰, corresponding to the Black Sea. A medium of higher salinity (30‰) was used in experiments with crosses, as the species had been earlier established to be euryhaline with an optimum salinity level close to 30‰ for sexual reproduction and vegetative growth for the Black Sea population. The crossing experiments involved 24 clones. To use at exponential
growth stage as an inoculum, the cultures were renoculated into a fresh medium every five to six days.

Cell sizes were determined under a Biolar-PI microscope (PZO, Poland) equipped with a ×12 eye-piece (lens) with an ocular micrometer of 1.60 μm scale interval when using ×40 water immersion objective and 6.92 μm at ×9 objective magnification. Additionally, cell sizes were estimated based on digital images using ImageJ v.1.43d software (http://rsb.info.nih.gov/ij). At least ten cells were measured in adherence to a random selection technique.

The images were taken using the digital Olympus C-5050, Canon PowerShort A95, and Canon PowerShort A640 cameras. Electron micrographs of our material were prepared using a Jeol JSM-6390 LA microscope (Japan).

RESULTS

The Black Sea biraphid pennate diatom alga C. scalaris is seen on the substrate in the form of singular motile cells with peculiar H-shaped chloroplasts (Fig. 1a); it occasionally lines up in rows but never forms mucilage tubes similar to the ones observed in Berkeleya Greville spp. [9]. Valves are linear, slightly lanceolate with medial expansion. Apical ends of valves are widely rounded or slightly capitate. Distal ends of raphe terminate internally (at the poles). Transapical striae run parallel over the entire length of valve, approximately 20 in 10 μm (Fig. 2). Cells of the alga contained in culture gradually decreased in size. Cases of abrupt size reduction were recorded. Cell sizes were measured in 12 clones over a period from 2 to 42 months. A total of 880 vegetative cells and 395 initial cells were measured. Sizes of cells in culture varied, ranging from 29 to 229 μm in length and 6.4 to 8.0 μm in width. Cells became sexually inducible after they decreased up to 114 μm in length. The lower limit of the size range in cells admissible for crossing fell within 30 to 42 μm. Clones that measured 29 μm in length were incapable of reproducing sexually. The number of chloroplasts contained in cells decreased with the cell sizes from 12–18 in initial cells to 3–4 in the smallest cells at the end of the life cycle (Fig. 3).

Sexual reproduction. C. scalaris exhibits both heterothallic and homothallic sexual reproduction. Heterothallism is easily seen if clones of different cell sizes are used for crossing and all gametangial pairs had different-sized cells.

The formation of gametangial cell pairs (parental cells where gametes are subsequently formed) was a first visual sign of entry into the sexual process (Fig. 1b). The motility of sexual partners allowed them to meet in mixed cultures even provided a low cell density. Gametangial cells were actively gliding relative to each other during the interaction. A mucilage track common for each pair was formed from the secreted mucilage (Fig. 1c); this was not observed in vegetative cells. Gametes and gametogenesis were observed to occur in mixtures of sexually compatible clones on the third or fourth day after inoculation. Each gametangial cell in a pair produced two elongated gametes with rounded ends (Fig. 1d). As chloroplasts prepared for division, they grouped, while migrating to the apical ends of the cell. Division of protoplast occurred in a transapical plane. No gamete rearrangement was observed; they were aligned along apical axis. Gametes were isogamous both morphologically and behaviorally. Their fusion occurred within the space between tightly appressed (to each other) parallel valves of two gametial cells. Syngamy is of a relatively short duration; less than 20 min elapse from the moment of recorded gamete movement to their fusion (Figs. 1e—1i). Gametes occasionally decreased in length prior to fusion but not necessarily. Gametes fused in pairs, which resulted in emergence of two zygotes (Fig. 1j). Syngamy is not always synchronized; there are cases when one pair of gametes has already fused, while fusion of the second pair is still delayed (Fig. 1k). A number of gametes did not fuse altogether; zygotes occasionally aborted as a result producing a single auxospore in a pair of gametangia (Fig. 1l). Auxospores expanded along the apical axis (Figs. 1m, 1n). When perishing, unfused gametes and aborted zygotes inflated and took the form of a sphere in which chloroplasts grouped in one position near the wall (Fig. 1o). Young auxospores were located between gametangial valves and did not differ much in shape from gametes and zygotes. The growing auxospores were located parallel to the parent valves. The auxospore growth was accompanied by the division of chloroplasts, whose quantity thus doubled relative to the total number produced by gametes (Fig. 1p). Initial cells escaped from the perizonium through active gliding. The perizonium is well developed with transverse bands visible under a light microscope (Fig. 1q).

Intraclonal reproduction in C. scalaris was rare and occurred in only two clones. Single occurrence of reproduction was recorded in monoclonal culture 5.0716-B. In the 5.0720–Z clone, intraclonal reproduction was recorded in mixtures with the Pacific 5.0630–F and 5.0630–D clones isolated from samples collected along the New Zealand coast. The Black Sea clone had a smaller cell size; homothallic reproduction was inferred, inasmuch as cells of a smaller size were exclusively involved in the process of sexual reproduction. At the same time, clone 5.0720–Z did not reproduce in monoclonal culture under similar conditions. The presence of the Pacific clones in the mixtures appear to have been a stimulus for intraclonal reproduction in 5.0720–Z clone. The remaining clones among those examined demonstrated heterothallism.
Fig. 1. Fertilization and formation of auxospores in Climaconeis scalaris (Brébisson) E.J. Cox. (a) Cells contain from 3 to 18 distinctive H-shaped chloroplasts dependent on their apical size. (b) Reproductively compatible cells form a gametangial pair. (c) Cells move relative to each other in pairs while forming a mucilage track; the chloroplasts concentrate in the proximity to apical poles; cells proceed to meiotic division. (d) Four gametes are formed in a pair of gametangial cells, which (e–i) fuse pairwise (time is shown in minutes and seconds), (j) producing two zygotes. (k) Fusion of one pair is occasionally delayed. (l–n) Auxospores expand; chloroplasts rearrange along apical axis, divide, and their number doubles. (o) Occasionally, gametes and zygotes are aborted. (p) Initial cells are two- to threefold longer than the parental; their size approaches a maximum for the species; contain the even number of chloroplasts twice as many as in the parental; the number of chloroplasts starts to decline in postinitial cells. (q) Initial cells escaping from the perizonium easily visible under a light microscope. (a, p) Light microscopy, bright-field illumination, (others) differential interference contrast. Scale bar 20 μm.
DISCUSSION

Diatom algae remain in a diploid state for a larger part of the life cycle; only gametes are haploid. Sexual reproduction is an obligate stage of the life cycle in the majority of diatoms [9, 10]. Studies into the crossing systems have shown that pennate diatoms exhibit predominantly heterothallic sexual reproduction [11, 12], which requires two clones. In a number of diatom species, the reproduction process can be accompanied by the behavioral and, occasionally, morphological distinctions between gametes produced by clones of different mating types as in, e.g., *Tabularia tabulata* (C.A. Agardh) Snoeijis, *T. fasciculata* (C.A. Agardh) D.M. Williams [13], and *Nitzschia longissima* (Brébisson) Ralfs [14]. In these species, cells of one clone produce active “male” gametes only, whereas cells of another reproductively compatible clone produce exclusively passive “female” gametes, which is the case of the so-called cis-anisogamy in terms of sex expression rather than types of mating. Anisogamy can similarly be observed in species with a single gamete formed in gametangium, e.g., biraphid diatoms in *Sellaphora* Mereschkovsky gen. [15] or particular *Cocconeis* Ehrenberg spp. among monoraphids [16]. A number of species have two gametes formed in each gametangial cell with one of them being active, while another is passive. This points at trans-anisogamy as in the case of, e.g., *Schizostauron* Grunow [17].

No differences were observed in *C. scalaris* in terms of morphology and behavior of gametes formed in the mating gametangia. The gametes were strictly isogamous morphologically and behaviorally. The pattern of sexual reproduction process in *C. scalaris* corresponds to the type IB2a in the Geitler classification [18]. A pair of gametangial cells each produced two functional gametes, the fusion of which resulted in the formation of two zygotes (Fig. 1j). Apical axes of gametangial cells and auxospores were parallel, which was well defined at early stages of auxospores’ bipolar expansion (Figs. 1m, 1n). A parallel position was apparently promoted by mucilage secreted at the stages when gametangial pairs were formed, which allowed the cells to stay at close proximity. A mucilage sheath (envelope), which could protect gametes from exposure to external factors, allow their close physical proximity and, therefore, enhance the likelihood of syngamy, was missing in *C. scalaris*, although it is found in more than 20 raphid diatoms genera, including *Craticula* Grunow, *Lyrella* Karajeva, *Placoneis* Mereschkowsky, etc. [20, 21].

Morphological and behavioral isogamy is rather common among raphid pennate diatoms. Based on the literature and our data, it takes place in at least...
15 genera, including raphid and araphid; anisogamous reproduction was described for 11 genera. The scheme of sexual reproduction of *C. scalaris* (IB2a) is shared by six diatom genera, all belonging to biraphid pennates. Phylogenetically *Climaconeis* is the most closely related to *Amphipleura* Kützing, *Berkeleya*, and *Frustulia* Rabenhorst genera [22]. Sexual reproduction observed in *Berkeleya micans* (Lyngbye) Grunow corresponds to the IB2a type [9], whereas *Amphipleura pellucida* (Kützing) Kützing and *Frustulia rhomboides* var. *saxonica* (Rabenhorst) De Toni had an inherently different pattern marked by type IA1αβ trans-anisogamy accompanied by the formation of copulation canals [23]. Evolutionarily, the sexual reproduction scheme is conservative; while being invariant, it ensures the unity of species and provides for transfer of genetic information through generations. The modification of its most important characteristics suggests a considerable evolutionary divergence as is observed in evolutionary lineage, extending from *Climaconeis* to *Amphipleura* and *Frustulia*.

The formation of copulation canals is typical in *Eunotia* Ehrenberg, *Neidium* Pfitzer, and *Nitzschia* Hassall spp. According to recent phylogenetic constructs, relying on the molecular data and time-calibrated based on emergence–extinction (first–last occurrence) of fossil diatoms [22], the evolutionary distance elapsed from the moment of these genera species divergence from ancestral forms to the present day amounts to approximately 30, 55, and 70 million years, respectively. According to the same data, genus *Berkeleya* has been in existence for a little over 75 million years; *Climaconeis* evolved into a separate genus approximately 70 million years ago; while *Amphipleura* and *Frustulia* originated shortly after, 55 million years ago. The occurrence of copulation canals and the associated trans-anisogamy in the latter can, therefore, be considered an evolutionary progress and, at the same time, a narrow specialization.

The cause of abortion of gametes and zygotes observed in *C. scalaris* is not well understood and warrants further study. Inbreeding and size of parental cells are known to affect the quantity of auxospores produced by the gametangial pair. Importantly, in *C. scalaris*, all clones were derived from the natural population and randomly isolated; the size of cells was 50–60% of the maximum species size, that is, they were at the early generative stage [24] and, therefore, there were no grounds for depressive phenomena.

H-shaped chloroplasts occur in representatives of multiple genera, namely, *Amphora* Ehrenberg ex Kützing, *Biremis* D.G. Mann & E.J. Cox, *Didymosphenia* M. Schmidt, *Gomphonema* Ehrenberg, and *Hantzschia* Grunow. *Climaconeis* spp. can commonly be distinguished based on this trait from the most closely related genus *Berkeleya*, featuring just two plate-like chloroplasts [5]. To distinguish between *Climaconeis* species, Reid and Williams [5] employed characteristics such as the number of chloroplasts in the cell. Notwithstanding, due to the aforementioned correlation between cell length and the number of chloroplasts, this trait should be utilized with caution, particularly as many diatom species are missing data with respect to the size ranges, corresponding to the complete life cycle and, consequently, to the possible number of chloroplasts in cell.

There can be only an even number of chloroplasts in initial cells due to their doubling in the growing auxospore (unless disturbed in the course of their division). As cells become smaller during a process of repeated mitotic divisions, the number of contained chloroplasts declines with time (there can be both even and odd numbers of the chloroplasts based on observations). The described regularity allows the assumption on the presence of a mechanism that regulates the number of chloroplasts in the cell, resulting from a decrease in its apical size during the life cycle. At the same time, no reliable correlation was detected.
between the sizes and the number of chloroplasts in initial cells (Fig. 3b).

Thus, fertilization in biraphid pennate diatom alga *C. scalaris* is allogamous, includes both heterothallic and homothallic sexual reproduction, and corresponds to the type IB2a in the Gaitler classification. The number of chloroplasts contained in the cell declines as the cell size becomes smaller during the life cycle.

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**COMPLIANCE WITH ETHICAL STANDARDS**

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals performed by any of the authors.

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