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Auxosporulation of *Licmophora communis* (Bacillariophyta) and a review of mating systems and sexual reproduction in araphid pennate diatoms

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SUMMARY

The auxosporulation of *Licmophora communis* is allogamous and dioecious. Pairing between sessile, short-stalked cells of compatible clones is followed by meiosis and gametogenesis, to form two gametes in each gametangium. The behavior of the gametes differs between the gametangia. In the male gametangium, the gametes detach from the frustule, round up, and migrate out of the gametangium after its dehiscence at the broader, unattached pole. In the female gametangium, both gametes remain attached to the adjacent theca over almost their whole length and do not move. Plasmogamy therefore occurs within the female gametangium and this is where the zygotes are formed and remain. After fertilization, the zygotes detach from the thecae of the female gametangia, contract, and become ellipsoidal, before expanding parallel to the apical axis of the gametangium. We review the types of auxosporulation in other pennate diatoms and the systems used for classifying these. Dioecy and *cis*-type anisogamy (in which one gametangium produces active gametes and the other produces passive gametes), as in *L. communis*, are probably primitive within the pennate group (although there is no information on the *Asterionellopsis*–*Rhaphoneis* clade). However, size can also be restored in various araphid pennates by allogamous sexual reproduction involving the formation of only one gamete per gametangium, or in rare cases by automixis or (apparently) vegetative enlargement.

Key words: auxosporulation, Bacillariophyta, diatoms, dioecy, heterothally, *Licmophora*, life cycle, mating system, pennate diatoms, sexual reproduction.

INTRODUCTION

The first report of auxospores in the Licmophoraceae was presented by Rejngard (1885). He described and illustrated the development of two auxospores apparently from a single parent cell in *Licmophora dalmatica* (Kütz.) Rabenh. (as *Podosphenia dalmatica*). There appear to have been no further observations of auxosporulation for 90 years, apart from an isolated claim of asexual auxospore formation by Hustedt (1927–1966, part 2, p. 53, Fig. 579b). Then Kumar (1978) succeeded in getting enlarged cells in small-celled cultures of *L. juergensii* C. Ag. and *L. hyalina* (Kütz.) Grun. After adding extra amounts of $NaNO_3$ in f/2 medium, she observed that some cells shed their frustules, after which the protoplasts became rounded, swelled, and formed new enlarged thecae. In *L. hyalina*, the same process could be stimulated by continuous illumination. Kumar considered the rounded cells to be auxospores and suggested that they appeared after an autogamous sexual process.

The first description of allogamous sexual reproduction in *Licmophora* was presented by one of us (Mann 1982) in *L. gracilis* var. *anglica* (Kütz.) Peragallo, from observations of natural collections from the North Sea. Here, cells paired and produced two gametes apiece. The gametes behaved anisogamously, two migratory (active) gametes being formed by one cell and two stationary (passive) gametes by the other. Both zygotes were thus formed within the confines of the same gametangium (mother cell). The zygotes were at first spherical and then expanded parallel to each other and to the apical axis of the mother-cell in which they lay. In Geitler's (1973) scheme of auxosporulation patterns in the pennate diatoms, this method of sexual reproduction would be classified as type IA2. On the basis of Mann's data, Geitler (1984) referred *L. gracilis* to subgroup IA2a (physiological anisogamy in which both gametes from a gametangium are either passive or active and in which the gametes round up and become rearranged within the gametangium), but this was not strictly justifiable, because Mann did not describe gamete morphology. By analogy with chemical isomers, the type of behavioral anisogamy found in *L. gracilis*, where both active gametes are produced by the same gametangium, may be referred to (Mann 1982) as the *cis* type, in contrast to the *trans* type found in some

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Table 1. *Licmophora communis*: lengths of cells in clones 1–4 and of initial cells in a mixed culture of clones 2 + 3

Clone	Date of measurement	*n*	$\bar{x}$ (μm)	s (μm)	Range (μm)
1	25.5.96	10	30.95	0.16	30.5–31.0
2	25.5.96	10	30.05	0.50	29.0–31.0
3	25.5.96	10	28.85	0.41	28.0–29.0
4	25.5.96	10	31.25	0.26	31.0–31.5
2 + 3 (initial cells only)	07.6.96	20	62.75	4.33	56.0–72.0

raphid pennate diatoms, where each gametangium produces one active and one passive gamete [Geitler's (1973) type IA1].

Later, type IA2 auxosporulation was also found in *L. ehrenbergii* (Kütz.) Grun. (Roshchin 1986, 1989b, 1994a; Roshchin and Chepurnov 1994) and *L. abbreviata* C. Ag. (Chepurnov in Roshchin 1994a). Clonal cultures were used to study the reproductive behavior of these two species, which both proved to be heterothallic (dioecious). Thus, within single clonal cultures, no signs of sexual reproduction were observed. However, when clones of either species were inoculated in pairs into mixed culture, auxospores were sometimes produced in large quantities, but only in particular combinations of clones (Roshchin 1986, 1989b, 1994a; Roshchin and Chepurnov 1994; Chepurnov in Roshchin 1994a). Analysis of the results of these crosses and observations of reproductive behavior showed that clones were strictly either 'male' or 'female'. Cells of male clones always produced two active gametes in compatible crosses and female clones always produced passive gametes. In addition, A.M. Roshchin and V.A. Chepurnov (unpubl. obss) found that no sexual reproduction occurred within single clones of *L. gracilis*, *L. dalmatica* and *L. flabellata* (Grev.) C. Ag. from the Black Sea that were small-celled (relative to the size range known for each species) and so were likely to be below the size threshold for sexual reproduction (Geitler 1932; Drebes 1977a). It is quite possible therefore that these species too are heterothallic.

Thus, Roshchin and Chepurnov's studies of clonal cultures and Mann's (1982) observations of natural populations suggest that *Licmophora* has *cis* anisogamy (Geitler's type IA2 auxosporulation) and dioecy (diplogenotypic sex determination), which conflicts with the early report of Rejngard (1885) and the later studies by Kumar (1978), which indicate the auxosporulation is autogamous or asexual. It would be useful therefore to have extra evidence concerning auxosporulation in *Licmophora* species and to critically review previous data, much of which is inaccessible to most phycologists, having been published in Russian. A few years ago, we were successful in isolating and culturing a further species of the genus, *L. communis* (Heiberg) Grun. in Van Heurck, and were able to induce auxosporulation in it. The results of breeding experiments and observations of sexual reproduction are presented in this paper.

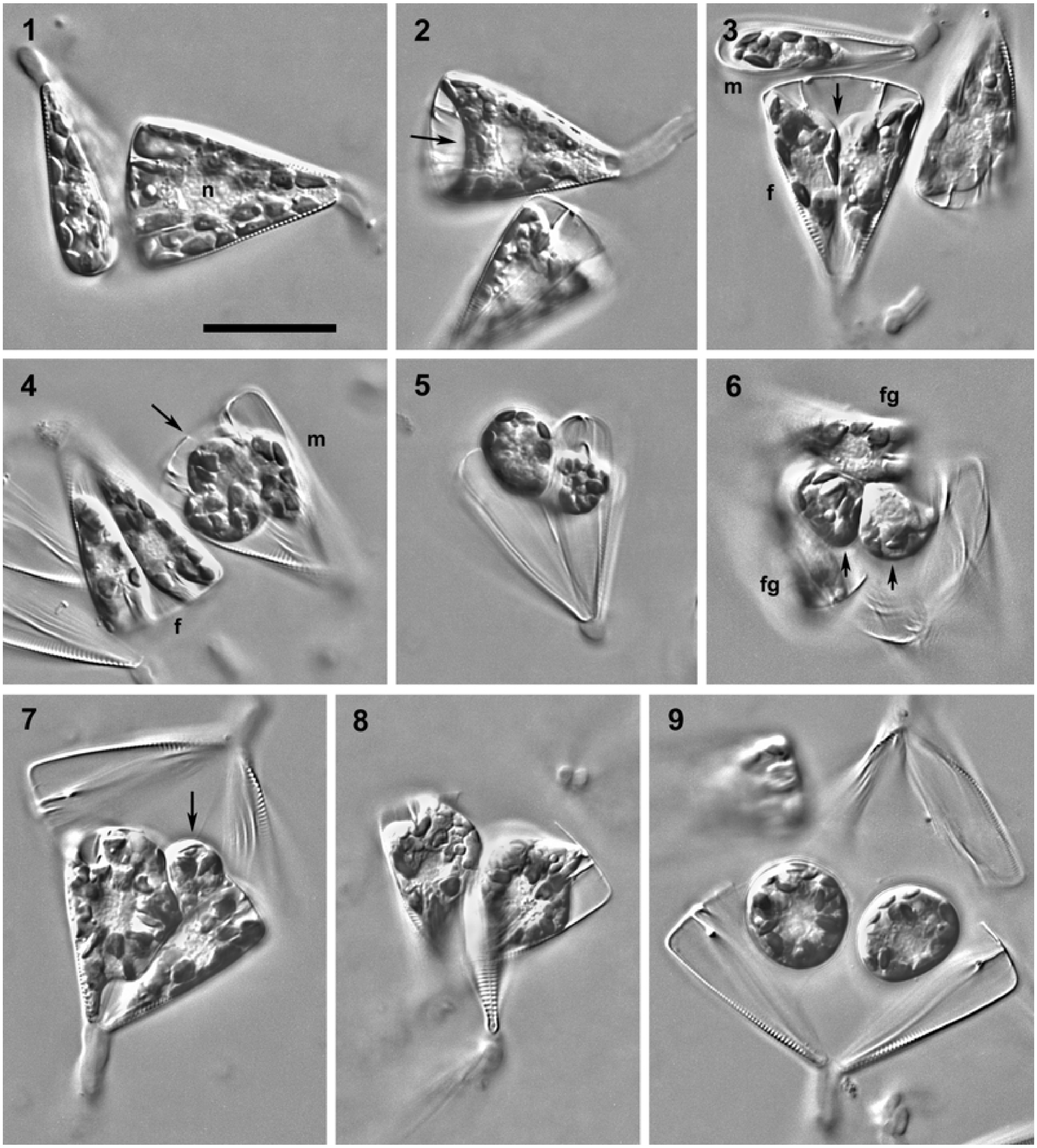
MATERIALS AND METHODS

A sample containing *L. communis* cells, which grew attached to the red alga *Dumontia incrassata* (O.F. Müll.) J.V. Lamour., was collected on 6 May 1996, from close to the low-tide mark in Ganavan Bay (Atlantic coast), near Oban, Scotland, UK, and transferred to the Royal Botanic Garden, Edinburgh. Two days after sampling, four cells of this species were isolated and grown as clonal cultures, using the marine enrichment medium described by Roshchin (1994a; see also Chepurnov and Mann 1997). The clones, and also the mixed cultures used for testing compatibility, were incubated in plastic Petri dishes at 20°C in dim light under 'cool white' fluorescent tubes, with a 12 : 12 h light–dark cycle. Reinoculations of cells into fresh medium were carried out every 4–5 days.

Observations of the cells were made while they were still in the Petri dishes (by examining them with a Zeiss Axiovert 135 inverted microscope), or by placing cover slips in the Petri dishes before inoculation, subsequently removing them with cells attached, and mounting them on microscope slides for examination with a Reichert Polyvar photomicrosope; the cover slips were ringed with petroleum jelly to prevent evaporation (Mann 1993b). Photographs were taken on Kodak Technical Pan film via the Polyvar photomicroscope, which was equipped with differential interference contrast (DIC) optics. Cell lengths were measured with the aid of a drawing attachment on a Karl Zeiss (Jena) microscope.

RESULTS

Cells multiplied rapidly in the laboratory (*c.* 2–3 cell divisions day^{-1}), so that by 12 days after inoculation, on 20 May 1996, the clonal cultures were already very dense. Individual cells were *c.* 30 μm long in all cultures (Table 1) and were broadly triangular in girdle view. Each contained many small lenticular or slightly elongate chloroplasts, which were scattered throughout the periphery of the cell, except near the central nucleus (Fig. 1). Cells were generally attached to the substratum singly, via a fairly short stalk (less than the length of the cell: Figs 1,2,7) or in small colonies. The stalks were bipartite, with a median line (visible when the cell was in girdle view) marking the boundary between the contributions of stalk polysaccharide made via the apical pore fields of the two thecae (Fig. 2, right).



Figs 1–9. *Licmophora communis*, clone 2 × clone 3: stages in gametogenesis and plasmogamy. 1. Paired cells in early meiotic prophase, containing many small chloroplasts and an enlarged central nucleus (n). 2. Meiosis I: a wide cleavage furrow (arrow) cuts through the protoplast. Note also the bipartite nature of the basal mucilage stalk (right). 3. Following cleavage, the daughter protoplasts in the female gametangium (f, clone 3) remain attached to their respective thecae and press against each other along the plane of cleavage (arrow); in the male gametangium (m, clone 2), the gametes have contracted more strongly. 4. Mature gametes: the female gametes (in f) are still attached to their thecae, whereas the male gametes (in m) have rounded up and are beginning to migrate out of the gametangium, which has dehisced (the arrow marks the free end of the epitheca). 5. Male gametes moving out between the thecae of the dehisced gametangium: note that the female gametangium was distant from the male in this case and is not shown. 6. Just before plasmogamy: the male gametes (arrows) have vacated the male gametangium (below, right) and entered the female gametangium, where they lie between and in contact with the female gametes (fg). 7. Fertilization of the left-hand female gamete has been completed; the second male gamete (arrow) is fusing with the right-hand female gamete. Note the empty frustule of the male (clone 2) gametangium (top) and that the female gametes/zygotes are still linked to the thecae of the female gametangia over their whole length. Both gametangia are stalked. 8. Following fertilization, the zygotes begin to contract away from the thecae of the gametangia. 9. Contracted, ± spherical zygotes with central paired haploid nuclei (not visible) lying within the gaping thecae of the female gametangium; empty male gametangium at top. Scale bar = 20 μm.

Table 2. Presence (+) and absence (–) of auxospore formation in mixed cultures of the four clones inoculated in pairs; the experiments were repeated three times

Clone	1	2	3	4
1	–			
2	+	–		
3	–	+	–	
4	–	+	–	–

No sexual reproduction occurred within any of the four clones. On 20 May, the clones were inoculated in pairs in all possible combinations. The results of the crosses (Table 2) show that all four clones were within the sexual size range, because each of them became sexualized in at least one mixed inoculation. In all mixed cultures where sexual reproduction occurred, auxospores appeared in large numbers. Sexual reproduction took place in any paired combination involving clone 2. Cells of clones 1, 3 and 4, however, did not mate among themselves. These experiments were repeated twice more, with the same result.

In all successful mixed cultures where sexual reproduction occurred, we observed the same allogamous sexual process. Cells of different clones paired while each was still attached to the substratum by a mucilage stalk (Figs 1,7,12). Generally, cells were close enough to touch (Figs 1,2), but we also observed a few cases (e.g. Fig. 12) in which paired cells were more distant (sometimes by more than the length of the cell) and apparently could not have been in contact when sexualization occurred; plasmogamy could nevertheless occur in such cases.

During meiotic prophase (Fig. 1), the nucleus remained central and swelled. Meiosis I was accompanied by cytokinesis and a marked contraction of the daughter protoplasts, so that they no longer occupied the whole lumen of the frustule (Figs 2,3). The daughter cells underwent meiosis II and became gametes. Immediately after cytokinesis, the parent frustule was intact and the gametes in both daughter cells remained attached to the interior of the valve. Later, the two thecae separated at the broader pole and in one gametangium (the 'male') the two gametes detached from the parent cell valves (Fig. 4), rounded up, and migrated out towards the other gametangium (Fig. 5). Movement of the protoplasts was slow and involved translocation of the whole rounded cell; the mechanism was unclear but it appeared to require contact with surfaces, such as the thecae of the gametangia. Meanwhile, the gametes in the other ('female') gametangium remained attached to the valves and did not move (Fig. 4). Plasmogamy occurred when the active male gametes entered the female gametangium and fused with the passive female gametes (Figs 6–8). The zygotes then detached from the female gametangial thecae and contracted, becoming spherical (Fig. 9). Although it was not visible in our preparations, mucilage was probably present around the zygotes, because they appeared held in place in characteristic positions, one on either side of the median valvar plane of the female gametangium (i.e. side-by-side in the girdle views shown in Figs 9,10); such mucilage was detected in *L. gracilis* by Mann (1982).

Later, the zygotes (auxospores) began to expand (Fig. 10). Expansion took place at both ends of the auxospore and was unequal, the end nearest the base of the gametangium being slightly narrower; however, the expanding auxospores were less strongly heteropolar than the gametangia (Figs 11,12). Karyogamy took place at an early stage, fused diploid nuclei (with two nucleoli) being visible, for instance, in the auxospores shown in Fig. 12. Few significant variations in auxospore formation were observed, except that sometimes one of the auxospores of a pair developed more slowly or not at all (Fig. 11). In one case, we found a triradiate auxospore in a mixed culture of clones 2 and 3, accompanied by a large isodiametric but unexpanded auxospore and a normal auxospore (Fig. 13); these auxospores appeared to have resulted from aberrant fusions among the gametes of at least three gametangia.

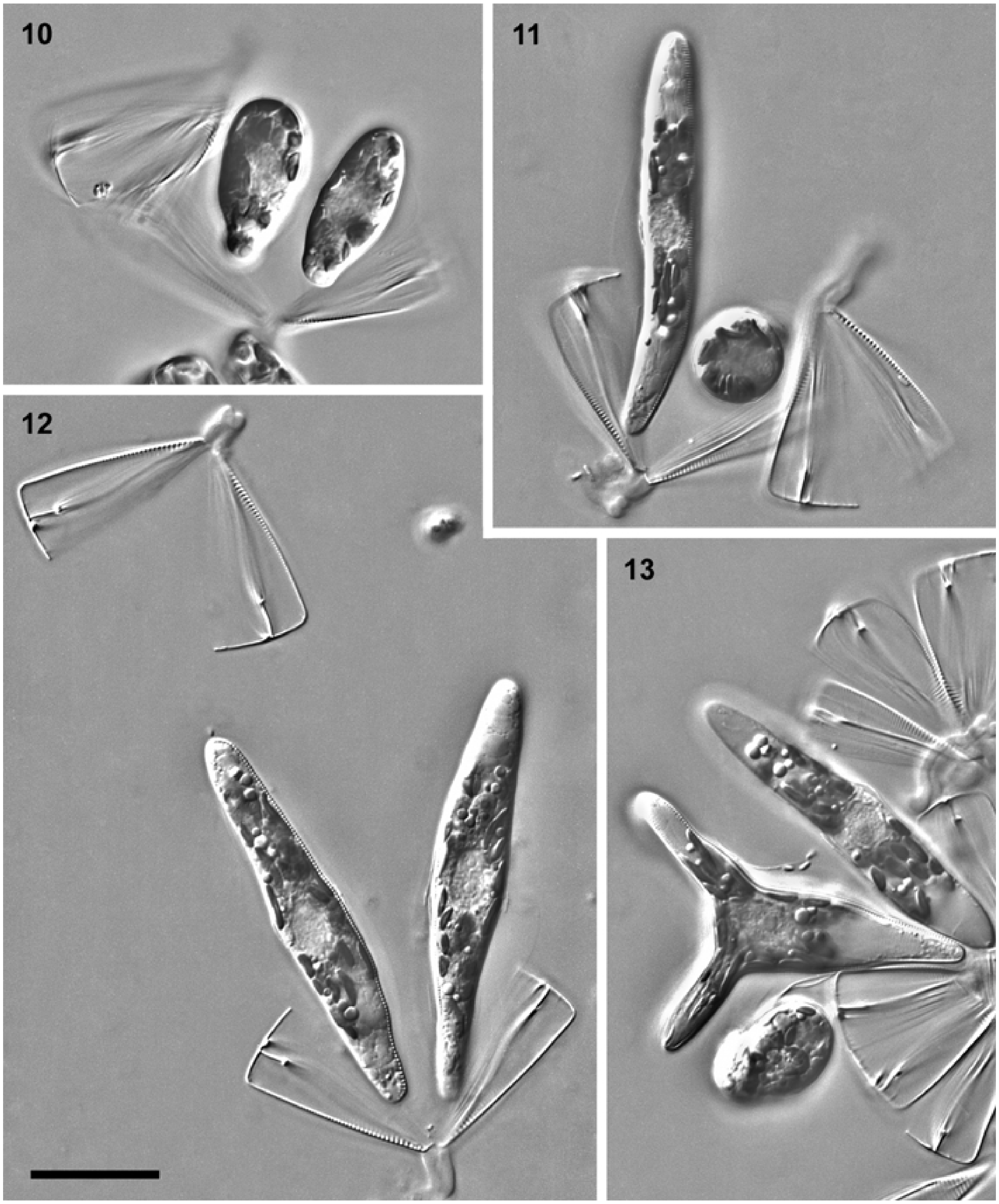
Initial cells were formed within the expanded auxospores (not illustrated), which were approximately twice as long as the gametangia, or slightly more (Table 1), measuring up to 72 μm. Because the auxospores were often somewhat irregular in shape (Figs 11,12), the initial cells and their immediate descendants were also more variable in morphology than smaller vegetative cells and gametangia.

Unfortunately, the clones studied were very similar in size, the length ranges either overlapping or being contiguous, although the means differed (Table 1). Nevertheless, we are confident that cells of clone 2 always produced male gametes in mixed cultures and cells of clones 1, 3 and 4 produced female gametes. In mixed cultures of clones 2 + 1 and 2 + 4, the gametangia that produced active gametes were in most cases smaller than the gametangia that produced passive gametes and were never larger. In mixed cultures of clones 2 + 3, the active gametes were nearly always formed by larger cells and never by smaller. The most parsimonious explanation of these observations are that clone 2 always produced active, male gametes. Thus, the results of crossing experiments (see Table 2) and comparisons of lengths of gametangia show that *L. communis* is dioecious.

DISCUSSION

Auxosporulation in *Licmophora*

Licmophora communis is the third species of *Licmophora* in which breeding behavior has been studied using clonal cultures. Like *L. ehrenbergii* (Roshchin 1986,



Figs 10–13. *Licmophora communis*, clone 2 × clone 3. 10. Early auxospore expansion. 11. Fully expanded auxospore and unexpanded non-viable zygote. Note empty male gametangium at right. 12. Two fully expanded auxospores; the initial epivalve is present in both (visible in section in left-hand auxospore). Note the distance (more than a cell's length) between the male and female gametangia in this case. Both gametangia are stalked. 13. Formation of a normal auxospore (top), a triradiate auxospore (probably resulting from the fusion of three gametes), and a large but unexpanded zygote (bottom). These appeared to have been produced by plasmogamy between gametes

1989b; Roshchin and Chepurnov 1994) and *L. abbreviata* (Chepurnov in Roshchin 1994a) from the Black Sea, *L. communis* is characterized by type IA2 auxosporulation, which involves *cis*-type behavioral anisogamy, and by dioecy. In addition, type IA2 auxosporulation has been reported in *L. gracilis* var. *anglica* (Mann 1982), but this report was based on natural populations and so the mating system is unknown. In other species, the data are more fragmentary, but most or all can be interpreted as representing the same kind of sexual behavior.

Rejngard (1885; pl. 11, fig. 14) illustrated two auxospores of *L. dalmatica*, lying between the thecae of a single cell and reported that this configuration is commonest for this species. He also found cases where only one auxospore was present within the open frustule of the mother cell but he noted that, in pairs of auxospores, one of them sometimes separated very early and fell off (*op. cit.,* pl. 11, fig. 15), so that isolated observations of late stages might lead to the incorrect assumption that only one auxospore had been formed. Rejngard also considered, however, that in a very few cases, a mother cell does truly give rise to only one auxospore. Observations of gametangia like those of *L. communis* in Fig. 11, where the male gametangium is distant from the female, suggest that Rejngard probably described only the female gametangia and did not notice, or could not interpret, the empty frustules of the male gametangia. It is very common that, in natural collections, the frustules of male *Licmophora* gametangia are lost soon after plasmogamy (see, for example, Mann 1982). Thus, the simplest interpretation of Rejngard's description and illustrations is that they represent an incomplete account of type IA2 auxosporulation. In addition, our suggestion is supported by the brief remark by Honeywill (1998), in relation to auxospore formation in *L. dalmatica* (not illustrated), in which she mentioned that 'the details correspond with the observations described by Mann (1982) and Roshchin and Chepurnov (1994)', that is those of *L. gracilis* var. *anglica* and *L. ehrenbergii*, respectively.

The process described as auxosporulation by Kumar (1978) in *L. juergensii* and *L. hyalina* looks very different from the patterns of auxosporulation found in other *Licmophora* species. Kumar maintained cultures (perhaps clonal) of *L. juergensii* and *L. hyalina* for long periods (10–12 months), during which the cells became very small and 'lost their characteristic wedge-like shape and became box-like. This change in shape always coincided with the loss of the formation of mucous stalks …'. She observed only vegetative multiplication of the cells, except after a change in growth conditions, involving addition of extra sodium nitrate to the medium or a change to continuous illumination, when some cells were induced to dehisce: the contents of the cells rounded off and the thecae of the mother cell were shed. Then, after some enlargement, new frustules were formed and the newly enlarged cells regained the ability to produce mucilage stalks. No pairing occurred and Kumar considered that the 'auxospores' were probably formed following autogamous sexual reproduction; however, nuclear processes were not studied and therefore no evidence was provided of meiosis or karyogamy.

We consider that there is a more likely interpretation of Kumar's observations than autogamous auxosporulation. Firstly, if it were auxosporulation, it is very strange that the 'auxospores' remained spherical and that bipolar expansion, known in other *Licmophora* species and present almost universally in other pennate diatom auxospores (the principal exceptions we know are in the surirelloid diatoms, which have highly modified symmetry and either exhibit unipolar auxospore expansion or achieve size restitution by simple longitudinal addition, e.g. Mann 1987, 2000), did not occur. Secondly, in Kumar's illustrations, the newly formed cells (a few divisions after expansion) were around 20 μm long in *L. juergensii* and no longer than 40 μm in *L. hyalina*. As is well known, auxosporulation is the mechanism by which maximal or nearly maximal cell size is restored (e.g. Geitler 1932; Drebes 1977a), but the enlarged cells illustrated by Kumar are much shorter (especially in *L. juergensii*) than the largest cells of these species found in nature — cells of *L. juergensii* can be 110 μm in length and those of *L. hyalina* 60 μm (Hustedt 1927–1966). The process described by Kumar as sexual (albeit autogamous) auxosporulation is therefore more likely to be vegetative cell enlargement, which is known from a variety of centric and pennate species (e.g. von Stosch 1965; Roshchin and Chepurnov 1992; Roshchin 1994a). Vegetative cell enlargement provides partial restoration of the cell size, without bipolar expansion: 'vegetative Zellvergrösserung kommt zustande durch Wiederbeschalung total oder partiell aus der Mutterzelle ausgetretener und dabei durch ihr osmotisches System aufgeblähter Protoplasten mir anschliessenden Zellteilungen, die den verbreiterten Klon etablieren' (von Stosch 1965; p. 27). In some diatoms, this process can be stimulated by changing the light or culture conditions, but it can also occur spontaneously (von Stosch 1965), especially in small cells.

During sexual auxosporulation, *Licmophora* species seem to be uniform in being dioecious. Direct evidence for this is available for *L. ehrenbergii* (Roshchin 1986, 1989b, 1994a; Roshchin and Chepurnov 1994), *L. abbreviata* (Chepurnov in Roshchin 1994a) and now *L. communis*. Long-term monoclonal cultures of *L. dalmatica*, *L. flabellata* and *L. gracilis* made by one of us (V.A. Chepurnov), which were all grown until the cells were small and hence likely to have been within the sexual size range (cf. Drebes 1977a), showed no signs of auxosporulation, implying that these species too are dioecious. If our interpretation of Kumar's data is correct, then *L. juergensii* and *L. hyalina* are probably also dioecious, because here too, no sexual reproduction occurred in monoclonal cultures, despite the small size of the cells.

Methods of sexual reproduction in araphid pennate diatoms

The most primitive method of sexual reproduction known at present in the araphid diatoms appears (see also Mann 1993a) to be the *cis*-type of anisogamy reported by von Stosch (1958, 1962) in three *Rhabdonema* species (*R. adriaticum* Kütz., *R. arcuatum*

Kütz. and *R. minutum* Kütz.). Here, as in *Licmophora*, there are two types of gametangia, one producing active male gametes and the other producing passive female gametes, but in *Rhabdonema*, not only are the gametes different in their behavior and shape (as in *Licmophora*), they are also differentiated in size, because depauperating mitoses occur during the formation of the male gametes, as during spermatogenesis in centric diatoms (von Stosch 1954; Drebes 1977a; Round *et al*. 1990; Roshchin 1994a). The male gametes are thus much smaller than the female and von Stosch referred to *Rhabdonema* as being oogamous, although the male gametes are not flagellate, being more like the spermatia of red algae. No other pennate diatoms have yet been found to exhibit depauperating mitoses. However, molecular phylogenies indicate that *Rhabdonema* is not a basal lineage within extant araphid pennate diatoms. For example, 18S rDNA phylograms published recently by Kooistra *et al*. (2003) and Medlin *et al*. (2000) show that the *Asterionellopsis*, *Rhaphoneis* and possibly also *Striatella* lineages could predate *Rhabdonema*. Unfortunately, no information is available concerning sexual reproduction in *Asterionellopsis* or *Rhaphoneis*. We have grown *Rhaphoneis amphiceros* (Ehrenb.) Ehrenb. in culture but have not yet succeeded in inducing sexual reproduction, either in monoclonal or mixed cultures, even in small-celled clones. *Striatella* does not exhibit depauperating mitoses (Chepurnov in Roshchin 1994a), but its phylogenetic position needs further clarification (apart from the *Asterionellopsis–Rhaphoneis* clade, most of the basal nodes of the araphid pennates have no significant support in the 18S rDNA tree).

In almost all araphid pennate diatoms for which data are available (Geitler 1973, 1984; Round *et al*. 1990; Roshchin 1994a), allogamous sexual reproduction is present and involves either *cis* type behavioral anisogamy – type IA2 auxosporulation in Geitler's (1973) classification, in which two gametes are produced per gametangium – or the related type IIB, in which each of the paired gametangia produces only one functional gamete, one gametangium producing an active 'male' gamete and the other a passive 'female' gamete. The latter is known in *Grammatophora marina* (Lyngb.) Kütz. (Magne-Simon 1960, 1962) and appears to have been produced through reduction from type IA2 (this would be consistent with the molecular phylogeny of Kooistra *et al*. 2003; though again, we would emphasize that the branching order of araphid lineages is not robustly supported). Similar changes have occurred in *Navicula sensu stricto* (within the *N. cryptocephala* Kütz. complex: Geitler 1973) and in *Surirella (*see Geitler 1973; Mann 1987; p. 110).

At present, *Synedra vaucheriae* (Kütz.) Kütz. is the only exception. Here, only automictic auxosporulation has been recorded, not allogamous reproduction (Geitler 1958). However, the occurrence of automixis does not mean that allogamous sexual reproduction is absent in this species. Several cases are already known within the araphid group where, although *cis*-type allogamous reproduction is the normal form of auxosporulation, other methods of auxospore formation do occur in the same species: autogamy in *Synedra ulna* (Nitzsch) Ehrenb. (Geitler 1939a), apparent isogamy in *Tabularia tabulata* (C. Ag.) Snoeijs (Roshchin 1986, 1989a) and *Fragilaria delicatissima* Proshk.-Lavr. (Roshchin 1994a), and haploid parthenogenesis in *L. ehrenbergii* (Roshchin and Chepurnov 1994) and *L. abbreviata* (Chepurnov in Roshchin 1994a).

That araphid pennate diatoms are rather uniform in their mode of sexual reproduction – more uniform (e.g. Geitler 1973) than in the raphid diatoms to which the araphids gave rise, according to molecular data (e.g. Kooistra *et al*. 2003) – was already evident in the early 20th century. At that time, however, interpretation of their auxosporulation was erroneous, because araphid pennates were considered to be automictic or apomictic (see the review by Geitler 1936): this applies, for example, to the early descriptions of auxosporulation in *Rhabdonema arcuatum* (Smith 1856; Karsten 1898), *R. adriaticum* (Karsten 1899), *Grammatophora marina* (Karsten 1926), *Meridion circulare* (Grev.) C. Ag. (Lüders 1862). Later, these four species were re-investigated and found to be allogamous, with *cis*-type gamete fusion (see Geitler 1973). However, some cases remain where apomixis or automixis may be the most satisfactory explanations of the available data. In *Tabularia affinis* (Kütz.) Snoeijs (observations were reported under the name *Synedra affinis*) and *Tabellaria* sp., two auxospores are apparently produced by one mother cell (Karsten 1897, 1926; Geitler 1932), although it is possible that the male gametangium was simply overlooked, as we have suggested may have occurred in Rejngard's studies of *L. dalmatica*.

A further similarity between several araphid pennate diatoms concerns the orientation of the auxospores relative to the gametangia. In all *Licmophora* species investigated, as during dioecious reproduction in another stalked araphid diatom, *Tabularia (*e.g. Fig. 12, Karsten 1897, figure 10), the auxospores expand parallel to the apical axes of the gametangia, with their bases still enclosed within the gametangia. Essentially the same arrangement is found in *Rhabdonema*, *Grammatophora* and *Striatella*, except that here the zygotes migrate out of the thecae that contained them (as gametes) before fertilization, although they remain very closely associated with them. This process is illustrated in *Rhabdonema* by Lüders (1862, figures 11c–e) and in *Striatella* by Roshchin (1994a; figure 29е–з). The zygote begins to expand only when migration is complete and the zygote lies at the mouth of the theca that had contained it. After expansion, the fully developed auxospore and gametangial theca together have a characteristic 'T' configuration (e.g. von Stosch 1962, figure 1; Roshchin

1994a, figure 29к,п). Curiously, the same behavior and arrangement occur in the oogamous bipolar centric diatom *Attheya decora* T. West (Drebes 1977b). However, there is also some variation among araphid pennate diatoms. In the marine '*Fragilaria*' *delicatissima* (it is unlikely that this is congeneric with the freshwater *Fragilaria* species), the auxospores expand perpendicular to the gametangia (Roshchin 1994a), and in *Meridion circulare* (Geitler 1940), *Diatoma elongatum* (Tschermak-Woess 1973a), and apparently also in *Synedra ulna* (Geitler 1939b), the relationship between gametangia and auxospores seems to be inconsistent. The regularity of the orientation of the auxospores vis-à-vis the gametangia also breaks down during haploid parthenogenesis in male gametangia of *Licmophora ehrenbergii* (Roshchin and Chepurnov 1994) and during monoecious reproduction of male clones in *Tabularia tabulata* and *F. delicatissima* (Roshchin 1994a). However, the latter two species require reinvestigation, because the process of auxosporulation was not studied in detail.

Mating systems in araphid pennate diatoms

The first clear report of dioecious reproduction in araphid pennate diatoms was given by von Stosch (1958) for *Rhabdonema adriaticum* (see also Rozumek 1968); a little later, dioecy was found in *Grammatophora marina* (Stosch and Drebes 1964; footnote p. 211), but in both cases the published data were minimal. Then, dioecy was demonstrated in *Licmophora ehrenbergii* (Roshchin 1986, 1989b; Roshchin and Chepurnov 1994), *L. abbreviata* (Chepurnov in Roshchin 1994a), *L. communis* (this paper), *Tabularia tabulata* (Roshchin 1987, 1989a), *Fragilaria delicatissima* (Roshchin 1994a), and *Striatella unipunctata* (Chepurnov in Roshchin 1994a), using controlled crossing experiments. In addition, all of these araphid taxa are alike in that dioecious reproduction is accompanied by *cis*-type anisogamy (including the 'oogamy' of *R. adriaticum*) and there are strict differences between clones of opposite sexes in the morphology and behavior of the gametes, so that it is very easy to differentiate 'male' clones, whose cells obligately produce active gametes, from 'female' clones, whose cells obligately produce passive gametes. Within these eight species, dioecy is either obligate or it is combined with monoecy. However, where intraclonal reproduction does occur (in *T. tabulata*, *F. delicatissima* and *S. ulna*), it is rare and in one case (*S. ulna*), involves automixis. By contrast, von Stosch (1958, 1962) reported monoecious reproduction, by implication at a moderate or high frequency, in *Rhabdonema arcuatum* and *R. minutum* von Stosch (1958, 1962). However, no details were ever published, for example, as to whether comparisons were made between the frequency and characteristics of intraclonal, as opposed to interclonal reproduction.

Excluding *R. arcuatum* and *R. minutum*, sexual reproduction and breeding behavior are essentially the same in eight species of araphid diatoms, despite the fact that they represent six genera from four different families. Such uniformity of sexual behavior in such diverse diatoms suggests that diplogenotypic sex determination is the plesiomorphic state in all araphid diatoms, or at least in the araphid clade that gave rise to the raphid diatoms (because we do not know anything about auxosporulation in the *Asterionellopsis*–*Rhaphoneis* clade). A contrary viewpoint has been put to us in review (M. Mizuno). The argument centres on *Rhabdonema*. Here, the two species known to be capable of monoecy (*R. arcuatum* and *R. minutum*) produce two female gametes (eggs) per gametangium, whereas a third (*R. adriaticum*) produces only one. Because the two-egg type is ontogenetically simpler than the single-egg type (fewer meiotic products are destroyed), it seems likely that the two-egg type is the more primitive (see also Mann 1993a). By extension, it could thus be argued that *R. arcuatum* and *R. minutum* are more primitive than *R. adriaticum* and hence that the obligate dioecy of *R. adriaticum* is a derived state. Furthermore, the occurrence of depauperating mitoses during male gametogenesis in *Rhabdonema* could be regarded as compelling evidence for a basal position of *Rhabdonema* within the pennate diatoms. Thus, if *R. arcuatum* and *R. minutum* are the oldest surviving lineage of pennate diatoms, then the argument goes that the pennate diatoms must be primitively monoecious.

This argument is certainly plausible, but we would note in opposition: (i) that the presence of a more primitive type of gametogenesis in *R. arcuatum* and *R. minutum* does not mean that their mating system is also primitive; such characteristics could evolve independently; and (ii), as we have already noted, currently available molecular data do not support the view that *Rhabdonema* is basal within the pennate diatoms. The presence of depauperating mitoses in *Rhabdonema* and in centric diatoms may therefore be a homoplasy; alternatively, the loss of depauperating mitoses may be homoplasious within pennates.

Only more robust phylogenies, coupled with experimental studies of clonal cultures of a wide selection of araphid pennate diatoms can test these ideas. We would identify two priorities for further work: (i) studies of sexual reproduction in the *Rhaphoneis*–*Asterionellopsis* clade, which is the sister group to the clade containing *Rhabdonema*, *Striatella*, *Tabularia*, *Licmophora* and the raphid diatoms; and (ii) re-examination of the mating system in the other araphid diatoms already known to exhibit allogamous sexual reproduction involving *cis* anisogamy, viz. *Rhabdonema arcuatum*, *R. minutum*, *Synedra ulna*, *S. rumpens* Kütz., *S. amphicephala* Kütz., *Diatoma elongatum* (Lyngb.) C. Ag., *L. gracilis* var. *anglica* and perhaps *Meridion circulare* (Geitler 1973; Round *et al.* 1990).

Comparisons with raphid pennate diatoms

Dioecy has also been found in several members of the raphid group (Roshchin 1994a; Roshchin and Chepurnov 1999) and so it is quite possible that the most primitive raphid diatom was dioecious and exhibited *cis*-type anisogamy, like its araphid ancestors. This argument is supported by the apparent complexity of *cis*-type anisogamy, which requires differentiation in several different aspects of gametangium and gamete behavior, e.g. stimulation of sexual activity by cells (pro-gametangia) of opposite sex (e.g. Rozumek 1968), different patterns of gamete maturation and rearrangement in male and female gametangia, oriented movement of the male gametes, self- and non-self recognition between the male and female gametes, and so on.

Mating systems have been studied in detail in very few raphid species but it is already becoming obvious that they are very diverse. There are species, such as *Haslea subagnita* (Proshk.-Lavr.) Makarova and Karayeva (Roshchin 1991; Chepurnov 1993), *Nitzschia longissima* (Bréb. ex Kütz.) Grun. (Chepurnov in Roshchin 1994a), or *Seminavis* cf. *robusta* Danielidis and D.G. Mann (Chepurnov *et al*. 2002), in which the clones grown exhibited obligately dioecious behavior, but there are also species that are vigorously monoecious, such as *Sellaphora seminulum* (Grun.) D.G. Mann, studied in detail by Geitler (1932). There are still other raphid diatoms that combine monoecious and dioecious behavior ('monoecious–dioecious' species) in different ways, including *Achnanthes longipes* C. Ag. (Roshchin 1994b; Chepurnov and Roshchin 1995; Chepurnov and Mann 1997, 1999, 2000), *A. brevipes* C. Ag. var. *brevipes* (V.A. Chepurnov and D.G. Mann, in preparation), *A.* cf. *parvula* Kütz. (V.A. Chepurnov and D.G. Mann, unpubl. obss), *Nitzschia lanceolata* W. Sm. (Roshchin 1990, 1994a), and probably *Navicula pennata* var. *pontica* Mereschk. (Roshchin 1994a). In *Achnanthes longipes*, for example, in addition to unisexual clones of either sex, which are obligate outbreeders, there are clones that are able to mate with either type of unisexual clone, that is bisexual clones, some of which can reproduce monoeciously (Chepurnov and Mann 1997, 1999, 2000). Furthermore, dioecy can be linked with different types of gamete behavior. It can be associated with different types of anisogamy, such as Geitler's (1973) type IA2 auxosporulation (*cis*-type anisogamy) in *Nitzschia longissima* but type IA1 (with *trans*-type anisogamy, both gametangia therefore being alike in producing one active and one passive gamete) in *N. lanceolata*; and it can be associated with different types of isogamy, such as with type IB2 in *Haslea subagnita* (where the apical axes of the gametangia and auxospores are parallel), but with type IC (no consistent orientation of the gametangia and auxospores) in *Achnanthes longipes* and *A.* cf. *parvula*, and a type of behavior somewhat transitional between types IB2 and IC in *Seminavis* cf. *robusta* (Chepurnov *et al*. 2002). So, raphid diatoms seem to be much more diverse in their patterns of breeding behavior than araphid diatoms, which correlates with the enormous taxonomic and ecologic diversity of this group: the raphid clade outnumbers both the centrics and araphid pennates (both of which are paraphyletic: see, e.g. Medlin *et al*. 2000) in terms of species, and possibly also of genera.

Classification of the types of anisogamous reproduction in pennate diatoms

Thirty years ago, Geitler (1973) published a classification of auxosporulation patterns in pennate diatoms, which was the last of a series of refinements he made on similar classifications dating back to the 19th century (e.g. Smith 1856; Hustedt 1930). This scheme is still a good summary and accommodates most of what is known about the great diversity of methods of sexual reproduction in pennate diatoms. However, in some respects Geitler's classification needs refinement, including his treatment of type IA2 auxosporulation, in which two gametes are produced per gametangium and there is *cis*-type fusion of the gametes. Category IA2 was divided by Geitler into two subgroups, on the basis of the behavior and morphology of the gametes before fusion. Thus type IA2a was reserved for cases where there was 'Umlagerung und Abkugelung der Gameten' (rearrangement and rounding off of the gametes), whereas type IA2b was 'ohne Umlagerung, doch mit Kontraction der Gameten' (without rearrangement, but with contraction of the gametes) (Geitler 1973).

This simple subdivision of category IA2 has been called into question by observations of araphid pennate diatoms and the raphid genera *Mastogloia*, *Achnanthes* and *Amphora sensu* lato. Stickle (1986) described auxosporulation in *M. smithii* Thwaites ex W. Smith and demonstrated *cis* behavioral anisogamy. This was the first certain discovery of *cis* anisogamy within the raphid group, because Stickle argued cogently that Geitler's (1973) previous reports of type IA2 auxosporulation in *Berkeleya rutilans* (Trentepohl ex Roth) Grun. (based on observations by Tschermak-Woess 1973b; as *Amphipleura rutilans*) and *Craticula halophila* (Grun. ex Van Heurck) D.G. Mann (based on observations by Subrahmanyan 1947; as *Navicula halophila*) were erroneous. Stickle tried to classify the auxosporulation of *M. smithii* within one of the two Geitlerian subgroups with *cis*-type anisogamy, types IA2a and IA2b, but noted that whereas '*M. smithii* does indeed show re-arrangement of gametes as stipulated for category IA2a … the re-arrangement occurs only in one gametangium; … In fact *M. smithii* could equally well be assigned to category IA2b, where there is no re-arrangement of the gametes … Thus, some modification of Geitler's (1973) scheme may be necessary.'

Later, type IA2 auxosporulation was found by Mizuno (1994) in *Achnanthes javanica* f. *subconstricta* Meister. In this diatom, re-arrangement of gametes again occurs in only one of the paired gametangia, as in *M. smithii*, 'but whereas it is the active gametes that re-arrange in *M. smithii*, in *Achnanthes javanica* f. *subconstricta*, only the stationary ones re-arrange.' This difference too cannot be accommodated within Geitler's system and so Mizuno agreed with Stickle that modification of the Geitler scheme is necessary' (Mizuno, *op. cit.*).

Mizuno therefore divided category IA2 into four new groups, 'according to whether the gametes become re-arranged within the gametangia and, if they do, which gametes'. Each of Mizuno's four subgroups is characterized by the position of gametes within the gametangia and whether this changes during gametogenesis. The assumption is made that whether the gametes become re-arranged or not within the gametangia is a fixed characteristic of auxosporulation within a particular taxon or population. Our experience of *Licmophora* shows that there is indeed a strict rule concerning rearrangement of the gametes within 'female' gametangia: there is never any. However, in 'male' gametangia, the presence or absence of re-arrangement is not strictly determined and appears to depend on the exact positioning of the gametangia relative to each other. This is well illustrated, for example, in *L. ehrenbergii* (Roshchin and Chepurnov 1994). Here, if the paired gametangia are in contact and dehisce towards each other, so that plasmogamy is possible very soon after dehiscence (Roshchin and Chepurnov 1994, figure 1 a, б, в; see also Roshchin and Chepurnov 1999, figures 1 and 2), re-arrangement of the male gametes within the frustule is commonly absent. But when the male and female gametangia are oriented perpendicular to each other, or where the gametangia only partially dehisce, so that immediate fusion of the gametes is impossible and plasmogamy can only occur after prolonged movement of the active gametes, then re-arrangement of the active gametes will generally occur (Roshchin and Chepurnov 1994, figure 2б). Hence, observations of many auxosporulating cells are necessary before one can be confident about the characteristics of the 'male' gametangia and gametes.

The final point we would make relates to the fact that important aspects of auxosporulation sometimes cut across Geitler's categories. One of these is morphological differentiation, as opposed to behavioral or size differentiation, between the gametes in anisogamous species. Anisogamy occurs not only in type I diatoms, that is those producing two gametes per gametangium, but also in type II auxosporulation, where only one gamete is produced per gametangium. Furthermore, in both type I and type II examples of anisogamy, it is possible to make a distinction between two groups of species. Firstly, there are species in which the active and passive gametes are similar morphologically and differ only behaviorally, as for example in some 'races' of *Cocconeis placentula* Ehrenb. (Geitler 1973, 1982), *Sellaphora* species (Mann 1984, 1989; Mann *et al*. 1999), *Diploneis papula* (A. Schmidt) Cleve (Idei and Chihara 1988) or *Achnanthes javanica* f. *subconstricta* (Mizuno 1994). Secondly, there are species in which there is a clear and constant difference in morphology between the active and passive gametes, such as in the araphid pennates *Licmophora*, *Synedra*, *Tabularia*, *Fragilaria* and *Rhabdonema*, which are all of type I (except *Rh. adriaticum* in which the male gametangium produces two gametes and the female only one, so that this species cannot be fitted into either type I or II), and *Grammatophora*, which is of type II (Geitler 1973; Roshchin 1994a, etc. see above for references), and in the raphid diatoms *Nitzschia longissima* (Roshchin 1994a) and apparently also in *Pseudo-nitzschia* (Davidovich and Bates 1998), which are both of type I. In this second group, the active gametes always round off, unless they are prevented by early fusion, whereas the passive ones always remain attached to the thecae of the mother frustule until fertilization, although they sometimes contract (*S. ulna*, *N. longissima*); the passive gametes can have various shapes, depending on the species but they never become fully spherical, so that there is no problem in differentiating them from active gametes, even at the lowest magnifications of a light microscope.

Hence, there are alternative ways of classifying patterns of auxosporulation, depending on which characteristics are considered most important. The primary division made by Geitler and previous authors into types I and II emphasizes the number of gametes produced per gametangium. However, the presence of both types in some apparently natural groups, such as *Navicula sensu stricto* and *Surirella*, implies that the type I and II categories do not have major phylogenetic significance (i.e. characterizing higher-level taxa), although the number of gametes produced per gametangium must be subject to very strong selective pressures, because change from type I to type II halves the number of zygotes produced by a sexualized population; presumably, the change is tolerated only because of a compensating high level of fitness of the few zygotes that are produced. By contrast, certain types of auxosporulation, such as the particular form of *cis* behavioral anisogamy in araphid pennate diatoms that we illustrate here, or the particular kind of isogamy and auxospore development of *Eunotia* and its allies (Geitler 1951a, 1951b, 1951c, Hori 1993; Mann *et al*. in press), appear to have been retained in most members of a family or order. Thus although it is worth retaining and modifying Geitler's classification system (we will propose changes to both Geitler's scheme and Mizuno's additions in due course, after publication of new information on auxosporulation in *Achnanthes brevipes*), the system is only an aid to communication and its in-built hierarchy implies nothing about the pattern or direction of evolution (see also Mann 1993a).

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