Variation in the sexual behaviour of *Achnanthes longipes* (Bacillariophyta). II. Inbred monoecious lineages

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(*Received 14 April 1998; accepted 22 October 1998*)

This paper continues a series of studies of the allogamous raphid diatom *Achnanthes longipes*, which has a complex reproductive system combining unisexual, bisexual and monoecious behaviour. Following earlier work on the effects of inbreeding in the progeny of crosses between two unisexual clones, we studied the progeny of clones that are capable of a high degree of selfing in monoclonal culture ('monoecious clones'). Three generations of selfed progeny were examined. In addition, we investigated the F1 generation obtained after crossing two different monoecious clones. Monoecious clones produced monoecious or, more rarely, bisexual progeny, but did not give rise to unisexual progeny. As in inbred lineages made by crossing closely related unisexual clones, inbreeding in monoecious lineages leads to a reduction in the number of gametes formed by the gametangia, from two to one. Inbred clones exhibit marked inbreeding depression and only three inbred generations were possible in monoecious lineages. In the third, final inbred generation, monoecious sexual reproduction was initiated in monoclonal cultures but gametes rarely fused and none of the auxospores and initial cells that were formed were viable; this also occurred when the inbred clones were crossed with any of the other clones available. The significance of inbreeding and other aspects of the breeding system in *A. longipes* is discussed.

Key words: *Achnanthes*, auxosporulation, Bacillariophyta, breeding system, diatom, inbreeding depression, sexual reproduction

Introduction

The diatoms are unicellular organisms with highly developed sexuality. Most species that have been investigated are allogamous (sexual reproduction involves the fusion of gametes produced by different gametangia); only a few are autogamous, paedogamous or asexual (Geitler, 1932, 1973, 1984, 1985; Drebes, 1977; Round *et al.*, 1990; Mann, 1993). However, very few allogamous species have been studied in any detail and there is scarcely any information on breeding systems. Thus, we do not know in most cases whether, in nature, the gametangia are closely related, perhaps even members of the same clone, or whether there are mechanisms that prevent or hinder mating between close relatives.

The limited information available suggests that the two principal morphological groupings among diatoms – centrics and pennates – differ profoundly in their breeding systems. Centric diatoms seem to be basically monoecious, with oogamous sexual reproduction (Wiese, 1969; von Stosch *et al.*, 1973; Drebes, 1977; Roshchin, 1994a). The same clone can produce both male and female gametes, which are compatible and can give rise to viable auxospores. There can be a partial separation in time of the male and female functions, however, since it has been shown in several species that, as size reduction proceeds, eggs are produced first, then sperm (e.g. von Stosch, 1951, 1956; Drebes, 1977; contrast *Leptocylindrus danicus* Cleve: French & Hargraves, 1985). Such phasing of sexual development will tend to promote outbreeding, though this may not be why it has evolved (an alternative explanation is that it has to do with resource allocation and the sex ratio). Pennate diatoms appear to be fundamentally different. Recent studies of breeding systems in several marine and freshwater species suggest that pennate diatoms are primitively and basically dioecious, so that sexual reproduction does not occur, or occurs only infrequently, unless compatible clones are present. Alongside this, oogamy has been replaced by morphological and behavioural anisogamy within the araphid series and by various types of anisogamy and isogamy within the raphid series (Roshchin, 1994*a*; Roshchin & Chepurnov, in press; Chepurnov & Mann, in preparation). Our data suggest that monoecy, as demonstrated within clonal cultures of several species, e.g. by Geitler (1932), Wiedling (1948), Mizuno & Okuda (1985) and Mizuno (1994), is unusual and derived.

Although these generalizations seem to be valid, the more that the breeding systems of diatoms are studied, the more it becomes obvious that simple, obligate monoecy (for centric diatoms) or simple dioecy (for pennate diatoms) are far from universal. Within the centric series, *Coscinodiscus granii* Gough may be given as an example of more complex breeding behaviour. Among several clones isolated from the North Sea, Drebes (1968) found some

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that were predominantly unisexual, producing only one type of gamete, male or female. Occasionally, the male clones formed a few oogonia, however, and so the differentiation between clones was not absolute; Drebes therefore classified *C. granii* as 'subdioecious'. Later, however, Drebes isolated clones that behaved monoeciously (Drebes, 1974, 1977). A combination of monoecy and subdioecy has also been noted in Black Sea populations of *C. granii* (Roshchin & Chepurnov, in press). Another example of a centric diatom with a more complex breeding system is *Melosira moniliformis* (O.F. Müller) C.A. Agardh, from the Black Sea (Roshchin & Chepurnov, in press).

Among pennate diatoms, several species are now known that can reproduce both dioeciously and monoeciously (termed 'monoecious-dioecious' diatoms by Roshchin, 1994*a*). These include the araphid pennate taxa *Tabularia tabulata* (C.A. Agardh) Snoeijs (Roshchin, 1987, 1989*a*, 1994*a*, as *Synedra tabulata*) and *Fragilaria delicatissima* Proshkina-Lavrenko (Roshchin, 1994*a*), and raphid diatoms such as *Nitzschia lanceolata* W. Smith (Roshchin, 1990, 1994*a*), *Navicula pennata* A. Schmidt var. *pontica* Mereschkowsky (Roshchin, 1994*a*) and probably also *Achnanthes brevipes* C.A. Agardh var. *intermedia* (Kützing) Cleve (Roshchin & Chepurnov, 1993; Roshchin 1994*a*). The most elaborate breeding system found so far is in the marine cosmopolitan diatom *Achnanthes longipes* C.A. Agardh. Long-term studies of its life cycle and sexual behaviour in culture (Roshchin, 1984*b*, 1994*a*, *b*; Roshchin & Chepurnov, 1992; Chepurnov & Roshchin, 1995; Chepurnov & Mann, 1997) have shown that there are three types of clone: unisexual, bisexual and monoecious. Monoecious clones are panmictic. They will mate vigorously with any other type of clone – monoecious, bisexual or unisexual – but they also exhibit a high level of intraclonal reproduction (although this is never as vigorous as in interclonal crosses). Bisexual clones are also panmictic, but differ from monoecious clones in that intraclonal reproduction is infrequent or absent. Unisexual clones exhibit a low level of selfing, like bisexual clones, but they are not panmictic in interclonal crosses. Instead, clones will reproduce vigorously only in certain combinations, allowing the recognition of two mating types or 'sexes'. Mixed cultures of clones of the same sex are sterile (apart from the low background incidence of monoecious, intraclonal reproduction), while in crosses between clones of opposite mating type vigorous sexual reproduction takes place, as between unisexual clones and monoecious or bisexual clones. There is some evidence that the three types of clone differ in the size thresholds for *intraclonal* reproduction, the critical size being smaller in unisexual and bisexual clones than in monoecious clones (Chepurnov & Mann, 1997). As a result, monoecy is not only less intense in unisexual and bisexual clones, but takes place over a smaller fraction of the life cycle.

Recently, we performed mating experiments between clones of *Achnanthes longipes* with different breeding behaviour – monoecious, unisexual and bisexual – except, of course, between unisexual clones of the same sex (Chepurnov & Mann, 1997). A study of breeding behaviour in the progeny of such crosses may cast light on the genetic or developmental basis of sexuality in *A. longipes*, besides helping us to understand why natural selection has produced such a complex breeding system within one species, albeit a species that is unusually widespread, adaptable and plastic (von Stosch, 1942, 1965; Hendey, 1951, 1964; McIntire & Moore, 1977; Lange-Bertalot & Krammer, 1989). In this paper we describe the characteristics of inbred lineages derived from intraclonal mating in monoecious clones, and also the behaviour of clones obtained by mating two different monoecious clones. Information is already available about the behaviour of lineages arising from crosses between unisexual clones (Roshchin, 1994*b*; Chepurnov & Roshchin, 1995).

Material and methods

The clones of *Achnanthes longipes* used in these experiments were derived from the same 12 clones studied by Chepurnov & Mann (1997, table 1), which were isolated from microphytobenthos growing on rocks and small macrophytes in the shallow sublittoral zone of the Black Sea in April and May 1993. Their sexual behaviour was subsequently established through studies of monoclonal cultures and crossing experiments (Chepurnov & Mann, 1997). In the present paper we examined the inbred (intraclonal) progeny of the monoecious clone 6 and the progeny of crosses between clone 6 and clone 4, also a monoecious clone. The sexuality of progeny clones was determined by mating them with other clones of known sexuality (see Tables 3–6); these comprised clones 4–6 (monoecious), 7, 8 and 10 (unisexual clones of both sexes) and four pairs of sibling clones. The sibling clones were all bisexual, apart from (6 + 10)1A (which was unisexual), and were produced in the laboratory by crossing the monoecious clone 6 and the unisexual clone 10 and subsequently isolating four pairs of initial cells (these clones were numbered (6 + 10)1A, (6 + 10)1B, etc.).

Other pertinent methods, culture conditions and protocols for crossing experiments, etc., have been described by Chepurnov & Mann (1997). Mean cell lengths are based on measurements of 10 cells and variation is indicated by ± standard error of the mean, unless otherwise stated.

Results

I. Successive inbred generations of monoecious origin

The first inbred generation (MI)

Monoecious reproduction. In December 1993 we started our investigations of inbreeding in monoecious clones of *Achnanthes longipes* using clone 6, studied earlier by Chepurnov & Mann (1997). By then, cells of clone 6 were 19–32 μm long (mean = 24·1 μm, SD = 3·82). Auxosporulation took place at a low frequency (less than 2% of

Table 1. Characteristics of *Achnanthes longipes* clones obtained through inbreeding in a monoecious lineage derived from parental clone 6 (MI–MIII generations), and of clones obtained after crossing two natural monoecious clones, clones 4 and 6 (FI generation)

Clone		Date of isolation	Cell lengths[a] (μm) At isolation	Cell lengths[a] (μm) After abrupt cell size reduction	Monoecious reproduction
MI	(1A)	3.12.93	136 ± 1	60 ± 1	+
	(2A)	3.12.93	123 ± 1	36 ± 1	+
	(3A)	3.12.93	130 ± 1	40 ± 1	+
	(4A)	3.12.93	128 ± 1	54 ± 1	+
MII	(1A)	13.6.94	118 ± 1	33 ± 1	+
	(2A)	13.6.94	109 ± 1	73 ± 1	0
	(3A)	13.6.94	121 ± 1	76 ± 1	+
MIII	(1)	20.9.94	130 ± 1	46 ± 1	+
	(2)	9.12.94	150 ± 1	66 ± 1	+
	(3)	9.12.94	135 ± 1	88 ± 1	+
	(4)	9.12.94	128 ± 1	79 ± 1	+
	(5)	9.12.94	119 ± 1	55 ± 1	+
FI	(1A)	17.5.94	114 ± 1	49 ± 1	+
	(1B)	17.5.94	120 ± 1	58 ± 1	+
	(2A)	17.5.94	117 ± 1	58 ± 1	0
	(2B)[b]	17.5.94	119 ± 1		?
	(3A)	25.8.94	107 ± 1	46 ± 1	0
	(4A)	25.8.94	111 ± 1	46 ± 1	0
	(5A)	25.8.94	100 ± 1	43 ± 1	+

+, monoecious (intraclonal) reproduction occurs consistently and frequently; 0, intraclonal reproduction almost or entirely absent.
[a] Cell lengths are means ± SE ($n = 10$).
[b] This clone was lost before reaching the sexual size range.

cells) within cultures, but this was sufficient for isolation of initial cells. Auxosporulating pairs were chosen that exhibited the 'normal' type of sexual reproduction (Geitler, 1932, 1973), in which the gametangia each produce two gametes, which fuse to yield two zygotes and hence two auxospores. The characteristics of auxosporulation have been described previously (Rejngard, 1885; Karsten, 1897; Roshchin, 1994*b*; Chepurnov & Roshchin, 1995) and correspond to Geitler's type IC (Geitler, 1973).

Twelve pairs of initial cells were isolated. However, only one initial cell of each pair was viable. The other always died without dividing. Of the 12 clones established, four were used for further observations and numbered MI(1A)–MI(4A) (Table 1). In order to reach the sexually inducible size range sooner, all four clones were abruptly reduced in size, to *c.* 60 μm or less (Table 1), according to a method described previously (Roshchin & Chepurnov, 1992; Roshchin, 1994*a*; Chepurnov & Mann, 1997; see also von Stosch, 1965). Following this, each of the four clones began to produce auxospores monoeciously. Auxosporulation took place regularly after every subculturing, and was quite frequent. The highest frequencies occurred in clone MI(3A) in March 1994, when sexuality was induced in approximately 16% of cells (at a culture density of 272 ± 44 cells mm^{-2} on the bottom of 90 mm diameter Petri dishes, $n = 40$); MI(3A) cells were then 32 ± 1 μm in length. Cells of the MI clones formed tufts, in which the mucilage stalks were clustered close together, as in the monoecious clones of *Achnanthes longipes* studied previously (Chepurnov & Mann, 1997).

Various methods of sexual reproduction have been reported and illustrated in *Achnanthes longipes* (Roshchin, 1994*b*; Chepurnov & Roshchin, 1995; Chepurnov & Mann, 1997). Three methods occur more commonly than the others (Chepurnov & Roshchin, 1995; Chepurnov & Mann, 1997), namely the 'normal' type of reproduction mentioned above, the 'reduced' type and the 'intermediate' type. In the 'reduced' type, only one gamete is formed by each gametangium, so that only one zygote and hence only one auxospore is produced by each pair of gametangia (type IIA2a auxosporulation, according to Geitler's 1973 notation). In the 'intermediate' type, one of the gametangia in each pair produces two gametes while the other produces only one. Here, a single auxospore is formed per pair (as in the 'reduced' type) and there will also be one superfluous gamete. This mode of sexual reproduction corresponds to none of those recognized by Geitler (1973).

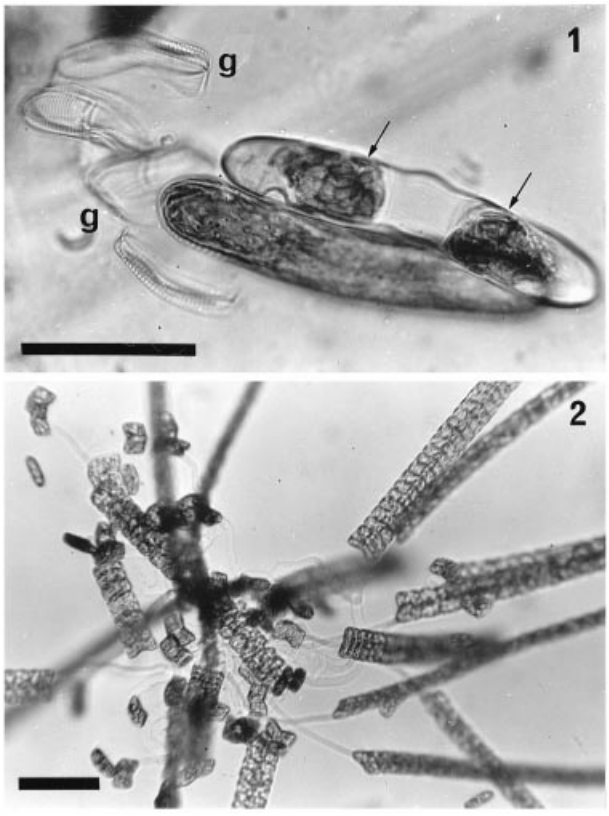
We estimated the relative frequency of these three types of behaviour, using as an index the number of auxospores produced by each pair of gametangia. The presence of two auxospores per pair must indicate the occurrence of the 'normal' type of reproduction. Where there is a single auxospore and no sign of any residual gamete, the 'reduced' type of auxosporulation has presumably occurred. The problem comes in those cases where two paired gametangia are accompanied not only by an auxospore but also by an aborted protoplast, since this could occur either following the 'intermediate' type of reproduction (in which case the aborted material represents the remains of the superfluous gamete) or following the 'normal' type of auxosporulation (if only one of the two zygotes developed while the other died before expansion). In view of this ambiguity, we united both variants within the same group, and hence Table 2 shows the relative numbers of 'two auxospores', 'one auxospore and an aborted body (zygote or gamete)' and 'one auxospore'. In 281 pairs of gametangia, drawn from all four MI clones (Table 2) the numbers of the different types of auxosporulation were 57:114:110 (20%:41%:39%). Careful study of those cases where an aborted product was present near the auxospore indicated that the 'normal' type of behaviour was generally more frequent than the 'intermediate' type.

Even during expansion, it was not uncommon for one auxospore to abort in each pair. The contents of such auxospores looked abnormal (Fig. 1) and they never developed into initial cells. In addition, very occasionally and only in clone MI(4), triradiate auxospores and initial cells were formed, like those illustrated by Hendey (1951, pl. I, fig. 1), Schmid (in Pickett-Heaps *et al.*, 1990, fig. 72d), and Chepurnov & Roshchin (1995, fig. 14).

Table 2. *Achnanthes longipes*: patterns of auxosporulation during the monoecious reproduction of clone 6 and its progeny in pure culture

Date	Clone	Cell length[a] (μm)	Type of auxosporulation: Type IC ('normal'): two auxospores[b]	Type of auxosporulation: One auxospore and an aborted body[c]	Type of auxosporulation: Type IIA ('reduced')[b]
7.12.93	6	24 ± 2	92	15	3
29.3.94	6	30 ± 1	42	6	3
21.4.94	MI(1A)	46 ± 1	13	25	21
23.5.94	MI(1A)	29 ± 2	2	1	24
10.2.94	MI(2A)	33 ± 1	3	8	1
15.2.94	MI(3A)	40 ± 1	3	10	6
22.2.94	MI(3A)	36 ± 1	11	20	13
10.2.94	MI(4A)	54 ± 1	7	13	8
22.2.94	MI(4A)	44 ± 1	9	10	5
14.3.94	MI(4A)	38 ± 1	2	12	16
22.3.94	MI(4A)	34 ± 1	7	15	16
25.7.94	MII(1A)	29 ± 1	2	8	31
28.9.94	MII(3A)	57 ± 2	1	3	14
11.10.94	MIII(1)	39 ± 1	0	0	21
3.1.95	MIII(5)	49 ± 1	0	0	18

[a] Measurements of cell length (mean ± SE, $n = 10$) were carried out within 10 days of the date on which observations of auxosporulation were made.
[b] The presence of two auxospores was unambiguous evidence of type IC auxosporulation. Likewise, the presence of one auxospore, with no evidence of aborted gametes or zygote, demonstrated that type IIA auxosporulation had occurred (see text and Geitler, 1973).
[c] A single expanded auxospore was accompanied by a degenerating protoplast, representing either an aborted zygote, or a superfluous gamete from the 'intermediate' type of auxosporulation (see text).



Figs 1, 2. *Achnanthes longipes*. Fig. 1. A pair of auxospores and their associated gametangia (g). The upper auxospore will abort: the cell has cleft into two protoplasts (arrows), which have contracted away from each other and are degenerating. Fig. 2. Formation of long chains of cells in loosely tufted colonies. Scale bars represent: Fig. 1, 50 μm; Fig. 2, 100 μm.

Interclonal crosses. Clones of the MI generation were crossed with each other and with the parental clone 6. The clones could mate in any pairwise combination but only at approximately the same frequency as during monoecious (intraclonal) reproduction, i.e. mating between the different clones was not vigorous (Table 3). In contrast, there was always abundant auxosporulation when MI clones were mated with monoecious, unisexual or bisexual clones that had been isolated from the Black Sea or obtained through laboratory crosses (see Table 3).

Other variants of sexual behaviour occurred rarely, such as haploid parthenogenesis, where a few gametes develop into auxospores without fertilization (see Geitler, 1979; Mann, 1994), and the formation of triradiate auxospores, as during monoecious reproduction.

The second inbred generation (MII)

Monoecious reproduction. Our first attempt to get clones of the second inbred generation (MII) was made in May 1994. Three initial cells were isolated that had been formed monoeciously from clone MI(1A); all were formed as a result of the 'reduced' type of sexual behaviour, involving the formation of only one auxospore by each pair of gametangia. None of them was viable, however, and so a second attempt was carried out using clone MI(3A). Of all the MI clones, this showed the most vigorous intraclonal auxosporulation. Four pairs of initial cells, formed after the 'normal' type of auxosporulation, were isolated in June 1994. In each pair, one initial cell proved to be non-viable, as in the monoecious progeny of

Table 3. *Achnanthes longipes*: results of crosses among MI clones, and between MI clones and various clones of known sexuality

		Clone			
Clone	Sexuality	MI(1A)	MI(2A)	MI(3A)	MI(4A)
MI(1A)	Monoecious				
MI(2A)	Monoecious	+			
MI(3A)	Monoecious	+	+		
MI(4A)	Monoecious	+	+	+	
4	Monoecious	++	++	++	++
5	Monoecious	NC	++	NC	++
6	Monoecious	+	+	+	+
7	Unisexual-1	++	++	NC	++
8	Unisexual-1	NC	++	NC	++
10	Unisexual-2	++	++	NC	++
(6+10)1A	Unisexual-2	++	++	NC	++
(6+10)1B	Bisexual	++	NC	NC	++
(6+10)2A	Bisexual	++	++	NC	++
(6+10)2B	Bisexual	++	++	NC	++
(6+10)3A	Bisexual	++	NC	NC	++
(6+10)3B	Bisexual	NC	NC	NC	++
(6+10)4A	Bisexual	++	++	NC	NC
(6+10)4B	Bisexual	++	NC	++	++

Unisexual-1, unisexual clone of sex 1; unisexual-2, unisexual clone of sex 2.
NC, cross not made; +, infrequent interclonal mating; ++, vigorous interclonal mating.

clone 6 (the MI generation), and one further initial cell died after two divisions. The other three initial cells began to multiply, so that three clones, designated MII(1A)–MII(3A), were available to study the second inbred generation (see Table 1). As before, abrupt size reduction was used to shorten the life cycle.

Having reached the sexual size range, MII(1A) and MII(3A) behaved as truly monoecious clones. In each, there was rather limited but regular auxosporulation and the formation of tufts of cells, as in the MI and other monoecious clones (Chepurnov & Mann, 1997). Clone MII(1A) continued to produce auxospores until the cells reached around 20 μm in length or even slightly smaller, when vegetative cell enlargement was observed. Clone MII(3A) started to reproduce sexually when the cells had reduced in size from 76 to *c.* 57 μm, but intraclonal auxosporulation ceased again when the cells reached *c.* 31 μm, considerably above the size at which cells usually cease to be able to reproduce sexually (*c.* 20 μm) or where vegetative cell enlargement starts to occur (15–18 μm). In addition, both clones exhibited two characteristics that we have never observed before in monoecious *Achnanthes longipes* clones. These were (1) the frequent formation, within the tufts, of ribbon-like colonies consisting of up to 30 cells (cf. Fig. 2); and (2) the regular attachment of cells to the water meniscus, where they multiplied successfully and reproduced sexually, just as on the bottom of the Petri dish. Clone MII(2A) was similar to the other two clones of this generation in its ability to form many-celled colonies but differed from them in that no monoecious reproduction occurred within the clone.

Table 4. *Achnanthes longipes*: results of crosses among MII clones, and between MII clones and various clones of known sexuality

		Clone		
Clone	Sexuality	MII(1A)	MII(2A)	MII(3A)
MII(1A)	Monoecious			
MII(2A)	Bisexual	+		
MII(3A)	Monoecious	+	+	
6	Monoecious	++	++	++
7	Unisexual-1	++	++	++
10	Unisexual-2	++	++	++
(6+10)4A	Bisexual	++	++	++

Unisexual-1, unisexual clone of sex 1; unisexual-2, unisexual clone of sex 2.
+, infrequent interclonal mating; ++, vigorous interclonal mating.

During the intraclonal auxosporulation of MII(1A) and MII(3A), the most common type of sexual reproduction was the 'reduced' type (Table 2). In 59 pairs of gametangia in these clones, the proportions of 'two auxospores' to 'one auxospore and an aborted body (zygote or gamete)' to 'one auxospore' were 5%:19%:76%. In the three pairs where two auxospores were produced per pair of gametangia (the 'normal' type of behaviour), one of the two auxospores appeared abnormal and was probably non-viable.

Interclonal crosses. The three clones of the MII generation were able to interact with each other sexually when grown together in pairs, but sexual reproduction was as rare as during monoecious reproduction (Table 4). However, when the MII clones were mated with various bisexual and unisexual clones, or with the monoecious clone 6 (the parental clone), vigorous auxosporulation took place (Table 4). Hence, MII(1A) and MII(3A), are true monoecious clones. Clone MII(2A), on the other hand, can be regarded as bisexual, since it was panmictic but did not reproduce monoeciously.

The third inbred generation (MIII)

Monoecious reproduction. MIII clones were derived from the monoecious clone MII(3A). Five initial cells were isolated in September 1994, but only one of these (MIII(1)) grew, the other four being non-viable. Later, in December 1994, four more clones of this generation (MIII(2)–MIII(5)) were isolated. All five MIII initial cells had been produced by the 'reduced' type of sexual reproduction. Just after cells of the five clones had been abruptly reduced in size (Table 1), transition to sexual reproduction was observed in MIII(1) and MIII(5). Intraclonal reproduction did not at first occur in the other three clones, MIII(2)–MIII(4), but took place once they had reduced further in size, to 55, 70 and 53 μm, respectively. All five clones, then, proved to be monoecious. In contrast to the MI and MII generations, the MIII clones did not form tufts of cells. Instead, they

Table 5. *Achnanthes longipes*: results of crosses among MIII clones, and between MIII clones and various clones of known sexuality

Clone	Sexuality	Clone				
		MIII(1)	MIII(2)	MIII(3)	MIII(4)	MIII(5)
MIII(1)	Monoecious					
MIII(2)	Monoecious	+				
MIII(3)	Monoecious	+	+			
MIII(4)	Monoecious	+	+	+		
MIII(5)	Monoecious	+	+	+	+	
6	Monoecious	++	++	++	++	++
7	Unisexual-1	++	++	++	++	++
10	Unisexual-2	++	++	++	++	++
(6+10)4A	Bisexual	++	++	++	++	++

Unisexual-1, unisexual clone of sex 1; unisexual-2, unisexual clone of sex 2.
+, infrequent interclonal mating; ++, vigorous interclonal mating.

remained solitary and usually produced longer and longer mucilage stalks as the density of the cultures increased.

In the MIII generation, the 'normal' and 'intermediate' types of reproduction were not found (Table 2). Each gametangium always contained only one gamete (the 'reduced' type of behaviour); in addition, it was very common for the gametes to fail to fuse and finally to abort. In the few cases where allogamous fusion of adjacent gametes took place, the zygotes usually failed to develop into auxospores. Occasionally, when auxospores appeared, they were of an abnormal shape and they never gave rise to initial cells. It was impossible to get a fourth inbred generation.

Interclonal crosses. The MIII clones were able to cross with each other in any combination (Table 5). As in the MI and MII generations, cells of different MIII clones mated with each other as infrequently as they mated with cells of the same clone. Again, however, when any of the five clones were grown together with unisexual, bisexual or monoecious clones that had been isolated from nature or produced through outbreeding in the laboratory, pairing between cells of different clones was vigorous (Table 5). It was noticeable throughout, whether the crosses involved close relatives (other MIII clones) or unrelated clones, that allogamous fusion of gametes and auxospore development were abnormal; as a result, there were very few zygotes, fewer auxospores and no initial cells, just as during intraclonal reproduction.

II. Progeny obtained after crossing two natural monoecious clones (FI generation)

The monoecious clones 4 and 6, isolated directly from natural populations, were crossed in May 1994. Their cells were then *c.* 26 and 37 μm in length, respectively, so that it was very easy to separate cases of pairing between cells of the same and different clones in mixed culture. Vigorous auxosporulation was observed in the mixed culture, as a result of sexual interactions between cells from the different clones; the background level of intraclonal reproduction was much lower. Here, the 'normal' type of behaviour was predominant, the proportions of the three main types of sexual behaviour being 76%:18%:6% (111 observations). From this mixed culture, two pairs of initial cells were isolated. All four cells, representing two pairs of sibling clones, started to multiply and were designated as FI(1A) and FI(1B), and FI(2A) and FI(2B) (Table 1). Unfortunately, following abrupt cell size reduction, clone FI(2B) was lost through experimental error. Later, in August 1994, three more pairs of initial cells were isolated from new mixed cultures of the same two clones, 4 and 6. In this case, however, only one initial cell of each pair survived. The three new clones were designated FI(3A), FI(4A) and FI(5A).

Monoecious reproduction. Three of the six clones exhibited an ability to produce auxospores monoeciously. Clones FI(1A) and FI(5A) started to reproduce sexually just after abrupt size reduction to below 50 μm (see Table 1), and

Table 6. *Achnanthes longipes*: results of crosses among FI generation clones (derived from crosses between monoecious parental clones 4 × 6) and between the FI clones and clones of known sexuality

Clone	Sexuality	Clone					
		FI(1A)	FI(1B)	FI(2A)	FI(3A)	FI(4A)	FI(5A)
FI(1A)	Monoecious						
FI(1B)	Monoecious	++					
FI(2A)	Bisexual	++	++				
FI(3A)	Bisexual	++	++	++			
FI(4A)	Bisexual	++	++	++	0		
FI(5A)	Monoecious	++	++	++	++	++	
6	Monoecious	++	++	++	++	++	++
7	Unisexual-1	++	++	++	++	++	++
10	Unisexual-2	++	++	++	++	++	++
(6+10)4A	Bisexual	++	++	++	++	++	++

0, clones incompatible; +, infrequent interclonal mating; ++, vigorous interclonal mating.

clone FI(1B) did so too once its cells had reached 50 μm. In each of the three clones, the 'normal' type of behaviour was much more frequent than the other two types. Thus, in clone FI(1B) the numbers of the three types of sexual reproduction were 21, 5 and 1. The other three clones of the FI generation produced by crossing clones 4 and 6 cross (clones FI(2A), FI(3A) and FI(4A)) never produced auxospores monoeciously.

All six clones of the FI generation were similar in their growth characteristics in culture. All were able to form tufted aggregations of cells, although the mucilage stalks were not clustered as close together as in the monoecious clones (Fig. 2: compare Chepurnov & Mann, 1997, fig. 2) and the tufts therefore seemed less dense. In addition, very long ribbon-like colonies containing up to several tens of cells were frequently observed within the tufts.

Interclonal crosses. The six clones of the FI generation proved compatible with each other and with other monoecious, unisexual and bisexual clones (Table 6), and sexual interactions were vigorous throughout. The only exception was the combination FI(3A) and FI(4A), where no interbreeding occurred; this result cannot be explained. The results of other crosses suggest, however, that clones FI(2A), FI(3A) and FI(4A) are all bisexual, since none exhibited appreciable monoecious (intraclonal) reproduction.

Discussion

The results confirm earlier observations (e.g. Chepurnov & Mann, 1997) that *Achnanthes longipes* possesses a complex breeding system, combining outbreeding and inbreeding in various ways. Clones exhibit particular patterns of mating, which are maintained throughout the sexual size range, allowing them to be classified as monoecious, unisexual or bisexual. The consistency of reproductive behaviour within clones suggests that their sexual characteristics are genetically determined, in contrast to those of many centric diatoms, in which clones are sequentially hermaphrodite, changing sex from female to male during size reduction (Drebes, 1977). Furthermore, we have now shown that the capacity for monoecious reproduction can be transmitted from generation to generation during inbreeding.

At present, we cannot suggest a genetic model to explain our results. Sex determination and the control of inbreeding seem to be much more complex in *Achnanthes longipes* than in various green algae that have been studied, such as *Pandorina morum* Bory (Coleman, 1959) or *Closterium ehrenbergii* Meneghini *ex* Ralfs (Kasai & Ichimura, 1990), but for the moment it is difficult to go much beyond description. This is partly because of the small number of clones we have been able to study: keeping the larger numbers necessary to determine segregation ratios is a major undertaking for which we do not have the resources. By contrast, in the dioecious diatom *Licmophora abbreviata* C.A. Agardh, sex determination is apparently simple and genotypic, since out of each pair of initial cells one is male and the other female (Chepurnov in Roshchin, 1994*a*). However, with the caveat that our observations apply to a very limited number of progeny, one interesting finding is that lineages tend to retain the same reproductive characteristics during inbreeding. Our inbred monoecious lineages did not give rise to any unisexual clones. Furthermore, the offspring from crosses between two monoecious clones (4 and 6) included monoecious and bisexual clones, but again no unisexual clones. In earlier experiments (Roshchin, 1994*b*; Chepurnov & Roshchin, 1995) we studied inbreeding in unisexual lineages, derived by mating sister clones derived from the FI, and subsequently the F2, of a cross between two unisexual clones isolated from the Black Sea. Here no monoecious clones were found in the first or second inbred generations, only unisexual and bisexual clones.

Most pennate diatoms produce two gametes per gametangium (Geitler, 1973) and this is probably the primitive state for the group (Mann, 1993). Generally, there is no variation in gamete number like that in *Achnanthes longipes* and gametes from the same gametangium rarely fuse. Our results also indicate that many araphid and raphid pennate diatoms are dioecious (heterothallic) and that this too is primitive in pennate diatoms (Roshchin & Chepurnov, in press; Chepurnov & Mann, unpublished observations). If this is correct, breeding systems like that in *A. longipes*, which allow or encourage inbreeding, must have evolved from outbreeding systems. A similar trend is reported in angiosperms (Stebbins, 1957; Barrett, 1989; Briggs & Walters, 1997) and genetic models predict that the evolution of inbreeders from outbreeders will generally be easier than the reverse (Lande & Schemske, 1985).

The behaviour of dioecious pennate diatoms (and of unisexual clones of *A. longipes*) during mating and copulation could be explained by assuming a cell–cell recognition system based on unipolar complementarity, the gametangia and gametes of different sexes (mating types) bearing different, complementary macromolecules involved in cell–cell recognition (e.g. see Hoekstra, 1987). Gametangia of the same sex would then be unable to mate and the two gametes of a single gametangium unable to copulate, since they would both bear the same, non-complementary recognition molecules; mating would only be allowed between gametangia of different sexes, bearing complementary macromolecules. This kind of mechanism has been demonstrated in other algae and is thought to be common in microbial mating systems (Hoekstra, 1987). However, there are considerable difficulties in explaining how organisms with unipolar complementarity could evolve the kind of selfing found in *A. longipes* and some other pennate diatoms, where mating is still prohibited between gametes from the same gametangium but is allowed between gametes from other, presumably genetically identical gametangia from the same clone. A change to bipolar complementarity (in which both complementary molecules are present on both

copulating cells) might be easy to achieve genetically and could explain the evolution of monoecy, but one would then expect the gametes within each gametangium to be able to fuse. This extreme kind of selfing does occur in diatoms, but it is very rare (having been reported only in a few habitually paedogamous species or races, such as *Cymbella aspera* (Ehrenberg) H. Peragallo *in* Pelletan: Geitler, 1956), perhaps because it leads very quickly to complete homozygosity for all genes that do not habitually segregate at meiosis I. By contrast, in diatoms producing only one gamete per gametangium, such as *Sellaphora* Mereschkowsky species (Geitler, 1932, 1957; Mann, 1989), a shift to bipolar compatibility would promote inbreeding without the possibility of intragametangial fusion and the catastrophic loss of heterozygosity it entails.

We suggest, therefore, that the evolution of inbreeding in pennate diatoms may well prove to be more common in taxa where only one gamete is produced per gametangium. Where it occurs in species producing two gametes per gametangium, loss of sexual differentiation may often be accompanied by breakdown or modification of the normal pattern of meiosis, cell division and gamete behaviour, leading to the peculiarities we have noted in *A. longipes*, and also to the bizarre pattern of behaviour in *Dickieia ulvacea* Berkeley *ex* Kützing, where we have observed paedogamy (*sensu* Geitler, 1973), undivided 'double gametes' and frequent multiple fusion of gametes, as well as allogamous auxosporulation of Geitler's (1973) type IC (Mann, 1994).

One of the principal effects of inbreeding is increased homozygosity, accompanied by display in the phenotype of recessive features, which were masked in the heterozygotic condition. Among these there are often deleterious mutations, which lead to inbreeding depression (e.g. Mayr, 1970; Dobzhansky *et al*., 1977; Solbrig & Solbrig, 1982; Maynard Smith, 1989; Muirhead & Lande, 1997). Intraclonal reproduction occurs at very low frequencies or not at all in unisexual and bisexual clones (Chepurnov & Mann, 1997). Here, therefore, inbreeding must be achieved experimentally by crossing closely related clones, derived from crosses between the same parents. In monoecious clones, fully inbred, selfed offspring can be produced. In both cases, inbreeding has marked effects on growth, reproductive potential and other characteristics, within a very few generations. In inbred monoecious lineages of *Achnanthes longipes*, abortion of zygotes and auxospores became more frequent, while auxospore development, stalk formation, and other aspects of growth and development became increasingly abnormal. It became progressively more difficult to obtain the next inbred generation and impossible to produce a fourth inbred generation. Similar negative effects of inbreeding have been reported in 'dioecious' lineages of *A. longipes* (Chepurnov & Roshchin, 1995). Though we have not quantified it, there is clearly a high degree of inbreeding depression. In addition, the trend towards the formation of only one auxospore per pair of gametangia will also tend to reduce the fitness of inbred lineages, whether or not this is caused primarily by disturbances to the mechanisms involved in sexual differentiation (as suggested above) or by a more general loss of vitality (resulting from the additive effects of many mildly deleterious genes). Furthermore, during monoecious reproduction involving the 'normal' type of auxosporulation, in which two auxospores are produced by each pair of gametangia, one of the auxospores is often not viable. This was true in all 12 pairs of initial cells used to initiate the MI generation from monoecious clone 6.

Nevertheless, although inbreeding has been shown to produce negative effects in *Achnanthes longipes*, the fact remains that close inbreeding (intraclonal reproduction via monoecy and interbreeding between sibling clones) is permitted and could potentially occur at a high rate in nature. By contrast, in most of the pennate diatoms we have studied, inbreeding between close relatives is prohibited in some way (Roshchin, 1994*a*; Chepurnov & Roshchin, 1995; Roshchin & Chepurnov, in press). The question arises, therefore, as to why natural selection has led to the particular balance between various forms of outbreeding and inbreeding found in *A. longipes*.

The advantages and disadvantages of inbreeding and outbreeding have been discussed extensively (e.g. Stebbins, 1950; Schmalhausen, 1939, 1969; Mayr, 1970; Maynard Smith, 1978, 1989; Schvartz, 1980; Briggs & Walters, 1997). Outbreeding continually generates new combinations of genes and can lead to an acceleration of evolution. There is often also a release of 'hybrid vigour' (heterosis), while the accumulation of deleterious mutations (Muller's ratchet) that occurs with asexuality or extreme inbreeding is avoided. On the other hand, outbreeding tends to break up successful gene combinations. Self-fertilization allows well-adapted genotypes to be replicated with little change, while in extreme habitats or at the margins of distribution, outbreeding may simply be impossible, through lack of suitable mates. In addition, the very same feature of selfing that leads to inbreeding depression, through the exposure of deleterious recessives to selection, can also be considered advantageous. 'Inbreeding populations … "purge" the mutations causing inbreeding depression more efficiently than outbreeding populations, and maintain fewer mutations on average' (Muirhead & Lande, 1997), although this occurs only above a threshold determined by mutation rate and inbreeding depression (Lande *et al*., 1994) and is ineffective against mildly deleterious alleles (Lande & Schemske, 1985).

Species, especially plant species, often combine outbreeding and inbreeding (e.g. Stebbins, 1950; Dobzhansky, 1951; White, 1954; Mayr, 1970). Mayr noted that 'the entire breeding system of outbreeders is so organized as to accumulate and preserve genetic variation giving a maximum of ecological plasticity and evolutionary flexibility, but at a price – the production of many inferior recombinants. An outbreeder may also be so well buffered that it stagnates evolutionarily. At the

other end is the extreme inbreeder which has found a lucky genotypic combination that permits it to flourish in a specialized environmental situation, but again at a price – an inability to cope with a sudden change of the environment. A species thus has the choice between optimal contemporary fitness combined with considerable evolutionary vulnerability and maximal evolutionary flexibility combined with the wasteful production of inferior genotypes. No species can combine the two advantages into a single system. Every species makes its own particular compromise between the two extremes'. This somewhat teleological argument is attractive, but not necessarily true. Quantitative models of evolution in seed plants predict that in many circumstances either complete outcrossing or complete selfing will be selected (e.g. Lande & Schemske, 1985; Lloyd, 1992) and surveys of outcrossing rates confirm a trend towards bimodality, with fewer species with intermediate outcrossing rates than would be expected by chance (Schemske & Lande, 1985; Barrett & Harder, 1996). On the other hand, a recent model shows how different breeding systems can be selected for in geographically or ecologically different parts of a species' range (Peck *et al*., 1998). Asexuality is demonstrated to be favoured at the margins of a species' range, since here sexually produced progeny will tend to be less fit than asexual or inbred individuals, as a result of 'contamination' by maladapted migrant genotypes, which are relatively more abundant towards the margins of distribution because of an overall decline in fertility as conditions become less optimal. Antonovics (1968) developed a similar argument to explain the occurrence of selfing populations of two grass species on soils contaminated by heavy metals, close to outcrossing populations on normal soils.

Among diatoms, a high degree of selfing probably occurs in nature in *Sellaphora seminulum* (Grunow) D.G. Mann, since not only are there no barriers to abundant sexual reproduction within clones, but successive inbred generations are possible with no obvious loss of vitality (Geitler, 1932, 1957, as *Navicula seminulum*). The same is probably true of *Gomphonema parvulum* (Kützing) Kützing (Geitler, 1932) and we have observed vigorous intraclonal auxosporulation in some demes of *Sellaphora pupula* (Kützing) Mereschkowsky, with no ill effects (Mann & Chepurnov, unpublished observations). Species such as *Licmophora ehrenbergii* (Kützing) Grunow, *L. abbreviata* and *Striatella unipunctata* (Lyngbye) C.A. Agardh, on the other hand, are obligately dioecious, with a genetic sex determination system (Roshchin, 1986, 1989*b*, 1994*a*; Roshchin & Chepurnov, 1994). This must considerably reduce the chances of inbreeding, as well as eliminating selfing. In *L. abbreviata*, when mating was attempted between sibling male and female clones derived from a single pair of auxospores, the progeny died after a few divisions (Chepurnov in Roshchin, 1994*a*); this high degree of inbreeding depression suggests that the species is predominantly outbreeding in nature. *Achnanthes longipes* seems to have an intermediate type of breeding system, allowing a mixture of inbreeding and outbreeding, and producing less extreme inbreeding depression than in *Licmophora*.

The contrast between the *Licmophora* species and *Achnanthes longipes* is interesting, since both are species of the marine littoral and sublittoral, forming extensive, dense growths on rocks or other solid substrata, to which they are attached by polysaccharide stalks; both exhibit marked changes in abundance during the year (Hendey, 1951, 1964; Daniel *et al*., 1987). There is no obvious reason for the stricter prohibition of selfing in *Licmophora*. At first sight, it is tempting to relate it to the lack of motility in *Licmophora*, so that dispersal is less effective and hence inbreeding will be inherently more likely, unless there are strict mechanisms to prevent it, such as obligate dioecy. However, this does not explain why the prohibition of inbreeding should be any less in *Achnanthes*.

There is some suggestion, however, that *Achnanthes longipes* is a particularly effective colonist and that its populations wax and wane more dramatically during the year than some other species of attached marine diatoms, including species of *Licmophora* and *Striatella* (Hendey, 1951, 1964). Hendey (1951) noted that *A. longipes* is one of the most important fouling diatoms, strongly resistant to the copper-containing paints used at the time to protect ships and other marine structures, and able to colonize surfaces quickly and build up very dense growths, especially in winter. In the Black Sea, *A. longipes* cells can be detected readily in the coastal plankton, at virtually any time of year (Proshkina-Lavrenko, 1955), and observations in culture show that *A. longipes* cells can rise within the culture vessel and colonize the surface film (cf. our observations of MII clones), where they grow and can even reproduce sexually (Roshchin, 1984*a*, *b*; Chepurnov, unpublished observations). Exactly how this is achieved is unclear. The cells may reach the surface via the walls of the culture vessel, but it is not impossible that the cells rise by becoming neutrally or even positively buoyant; further observations are necessary. Whatever the mechanism, however, the tendency for cells of some clones to migrate upwards, away from the substratum, can be expected to facilitate dispersal in nature. Not surprisingly, *A. longipes* is very widely distributed geographically and quite euryhaline (Makkaveeva, 1960; McIntire & Moore, 1977). Furthermore, few morphologically distinct forms or races have been described within it (e.g. see Hustedt, 1927–66; Proshkina-Lavrenko, 1950; VanLandingham, 1967), again suggesting the effectiveness of dispersal.

It seems obvious that, in environmental conditions optimal for growth of *Achnanthes longipes*, where the populations can successfully multiply and reach high cell densities, outbreeding will predominate. Outcrossing is enforced among unisexual and bisexual clones, while our experiments (Chepurnov & Mann, 1997 and see above) indicate that even monoecious clones outbreed by preference. *A. longipes* is motile and, at least in culture, unisexual and bisexual clones disperse themselves quite effectively across the bottom of the culture vessel.

Monoecious clones are more gregarious, but when cell densities are high, monoecious cells will often encounter members of other clones, with which they will be compatible, since monoecious and bisexual clones are panmictic. On the other hand, at particular times of the year, or in spatially limited or isolated populations, or at the margins of large populations, or wherever cell densities are low in relation to the mean distance cells move during the cell cycle, most encounters will be between cells of the same clone and the likelihood of successful outbreeding will decrease. For this reason, and perhaps also because of the possibility of 'dilution' by ill-adapted migrants from larger populations nearby (see above: Antonovics, 1968; Peck *et al.*, 1998), inbreeding may then become advantageous, although, if our clones are typical, enforced inbreeding will not be tolerated for long.

It may be significant that cells of the MII generation showed a particularly high tendency to rise to the surface in culture; this could represent an 'escape' mechanism for isolated, inbred lineages, allowing them to become temporarily planktonic. Among many tens of clones of *A. longipes* we have isolated from the Black Sea, only one was taken from the plankton, from a sample collected by A.M. Roshchin near Karadag in 1979, around 2 km from the coast (Roshchin, 1984*b*, clone 1). This clone proved to be monoecious and exhibited the strictly 'reduced' type of reproductive behaviour (Roshchin, 1984*b*), producing only one gamete per gametangium. Furthermore, its cells formed longer and longer mucilage stalks as the density of the culture increased (Roshchin, 1994*b*). Both features are characteristic of inbred lineages (see above; also Roshchin, 1994*b*; Chepurnov & Roshchin, 1995). It is theoretically possible that isolated populations and clones of *A. longipes* might be able to perpetuate themselves indefinitely without *any* sexual reproduction, since in culture cells can spontaneously enlarge vegetatively (Roshchin & Chepurnov, 1992; Roshchin, 1994*a*; in most pennate diatoms there is no such mechanism and size can only be restored sexually, via an auxospore). However, although this may occur, it is unlikely to be significant. Vegetative cell enlargement only takes place once cells have declined to less than 15–18 μm in length, and such tiny cells have yet to be observed in nature, despite extensive study of the species over many years (e.g. Hustedt, 1927–66; Hendey, 1951, 1964; Proshkina-Lavrenko, 1963).

Our study raises more questions than it answers, but we hope that, by attempting to address issues that diatomists usually ignore, we may stimulate new research into breeding systems in diatoms and into the frequency and distribution of inbreeding and outbreeding populations in nature. At present these are virtually unexplored subjects.

Acknowledgements

We are very grateful to Dr A.M. Roshchin for teaching and supporting one of us (V.A.Ch.) for many years, and for many useful discussions; Prof. Mike Sullivan and an anonymous reviewer for constructive comments; and Stephen Droop for assistance and discussion. Publication of this work was aided by INTAS (grants 93-3605 and 93-3605-ext).

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