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REPRODUCTIVE COMPATIBILITY AND rDNA SEQUENCE ANALYSES IN THE *SELLAPHORA PUPULA* SPECIES COMPLEX (BACILLARIOPHYTA)[1]

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We tested whether internal transcribed spacer (ITS) rDNA sequence differences are correlated with sexual compatibility in the *Sellaphora pupula* complex, a model system for investigations of the species concept and speciation in diatoms. The phylogenetic relationships among the demes and the systematic position of the genus within the raphid diatoms were also investigated. The division of clones of *S. pupula* and *S. laevissima* into groups, based on sequence similarities and phylogenetic analyses, resembled groupings based on sexual compatibility: A high ITS sequence divergence, making full alignment difficult or impossible, was found among clones whose gametangia do not interact, whereas there was little sequence divergence among interfertile clones. This is clearly consistent with the idea that "Z clades" exhibit less intraclade than interclade variation in ITS and, as comparisons of secondary structure models for the RECT and PSEUDOCAP clones showed, that there is an equivalence of "CBC" and Z clades in the rectangular and pseudocapitate demes of *S. pupula*, as earlier hypothesized for chlorophytes. Intraclonal, presumably intraindividual, variation in ITS was found in *S. pupula*, though with a degree of variation less than that found within a single Z clade; it was too minor to affect the interclonal relationships in the ITS phylogeny. *Sellaphora*, which appears monophyletic in 18S phylogenies, with *Pinnularia* and "*Navicula*" *pelliculosa* as its closest allies, may also include some species currently classified in *Eolimna*. The *S. pupula–S. laevissima* group began to diversify in or before the Miocene.

Key index words: **diatom; ITS rDNA; molecular systematics; paralogues; phylogeny; reproductive isolation; *Sellaphora*; species concept; Z clade**

Abbreviations: **CBCs, compensating base pair changes; ITS, internal transcribed spacer**

The *Sellaphora pupula* complex has become a model system for investigations of the species concept and speciation in diatoms (Mann 1984, 1989a, 1999, 2001, Mann and Droop 1996, Mann et al. 1999). Differences in the size, shape, and pattern of the valves that previous generations of diatomists thought had little or no systematic significance have been found to be correlated with barriers to successful sexual reproduction. Preliminary molecular sequence analyses, using 18S rDNA, have also showed significant differences between *S. pupula* demes (Mann 1999). We now extend these analyses to study the relationship between reproductive compatibility and genetic relationship as estimated from 18S rDNA and internal transcribed spacer (ITS) sequences.

The relationship between mating capacity and molecular sequence variation has been studied in the green alga *Pandorina morum* and related Volvocales. Coleman and coworkers hypothesized that organisms capable of interbreeding may show only minor sequence variation in their ITS rDNA, whereas between those that cannot interbreed, ITS sequence divergence is much higher (Coleman et al. 1994, Coleman and Mai 1997). Another correlation between sequence data and the capability to interbreed involves the secondary structure of the ITS-2 rDNA region (Mai and Coleman 1997). No compensating two-sided base pair changes (CBCs) (Gutell et al. 1994) occur between Volvocales strains that show gamete interactions; conversely, where CBCs do occur, strains are incapable of interbreeding (Coleman 2000). Coleman therefore

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suggested that the appearance of a CBC in ITS sequences marks the point in evolution where the mating genes of two organisms have diverged to such an extent that the organisms are no longer able to interact at the gamete level (Coleman 2000). No ITS data are yet available to test the hypothesis of a correlation between ITS sequence differences and sexual compatibility in diatoms.

ITS rDNA is a widely used marker for phylogenetic systematics in many groups of organisms, especially at the level of species or populations (Kooistra et al. 1992, 2002, Baldwin et al. 1995, Coleman et al. 1998). The small size of the ITS regions and the presence of conserved flanking regions facilitate PCR amplification and sequencing of many strains within a short time. Because of its relatively high evolutionary rates, ITS rDNA sequencing can be a powerful tool to resolve relationships at the level of species or populations (Baldwin et al. 1995; for diatoms, see Zechman et al. 1994). However, the extrapolation of ITS rDNA phylogenies to the organismal level may be affected by sequence variation among the different copies of rRNA cistrons present within the genome. ITS paralogues within a single genome, differing in their sequences (i.e. intragenomic ITS variation), have been found in a broad range of organisms (Ritland et al. 1993, Carranza et al. 1996, Tang et al. 1996). Consequently, some authors have even doubted the suitability of ITS rDNA for phylogenetic studies (Baldwin et al. 1995). Although ITS rDNA has apparently worked well as a phylogenetic marker in many green algae, it has failed spectacularly in other groups (Famà et al. 2000, Durand et al. 2002).

Sellaphora is a genus of raphid diatoms (Round et al. 1990) that was originally classified within *Navicula* because both have simple boat-shaped valves. *Sellaphora* was separated from *Navicula* by Mereschkowsky (1902) on the basis of differences in interphase chloroplast structure (one H-shaped chloroplast in *Sellaphora* vs. two strip-like chloroplasts in *Navicula*), but his classification was not accepted (Hustedt 1961–66). Further evidence supporting separation was given by Mann (1989b; see also Round et al. 1990), using data on the dynamics of chloroplast division and sexual reproduction. *Sellaphora* is certainly within the raphid diatoms (i.e. the class Bacillariophyceae *sensu* Round and Crawford 1990, Round et al. 1990). However, the phylogenetic position of the genus within that group is still unclear. Although an affiliation of *Sellaphora* with *Navicula* might be likely, based on the boat-like shape of the valve and uniseriate striae containing simple areolae, the little evidence available from 18S rDNA data (sequences of D. G. Mann et al. included in the phylogeny published by Medlin et al. 2000) suggests that *Sellaphora* is more closely related to *Pinnularia*, as had previously been implied by the inclusion of both within the suborder Sellaphorineae by Mann (in Round et al. 1990).

Sellaphora is currently a relatively small genus, containing approximately 30 species (Mann 1989b, Metzeltin and Lange-Bertalot 1998, 2002, Rumrich et al. 2000, Lange-Bertalot et al. 2003), although Round et al. (1990, p. 552) noted that many other *Navicula* species would probably need to be transferred into it. The best known species are the type species, *S. pupula* (Kütz.) Mereschk., *S. bacillum* (Ehrenb.) D. G. Mann, and *S. laevissima* (Kütz.) D. G. Mann. Within the *S. pupula* complex, several variants can be distinguished on the basis of valve morphology: The number of these is unknown but considerable (perhaps ~100). We refer to them as "demes" sensu Gilmour and Gregor (1939) and use informal names to distinguish between them (Mann 1989a, 1999, Mann et al. 1999). *Sellaphora pupula* demes are generally epipelic, and it is not unusual for two or more demes to live intermingled in the same lake. For example, in the best studied case—Blackford Pond, a small hypereutrophic lake in a public park in Edinburgh—seven demes have been found during 20 years of study; in Threipmuir Reservoir, near Edinburgh, the number is even higher. Both of these lakes are sources for the clones studied here.

Morphological distinctions between *S. pupula* demes are not usually qualitative, that is, they are not based on the presence or absence of particular characters but are a matter of degree (Mann 1999). However, members of different demes can have different mating systems (Mann 1999; unpublished observations), react differently to parasites (Mann 1999), and often cannot interbreed with each other (Mann 1984, 1989a, 1999, Mann and Droop 1996, Mann et al. 1999). For example, "capitate" clones and demes are capable of interbreeding, even when isolated from sites many thousands of kilometers apart, but they cannot successfully mate with clones from "rectangular" demes growing in sympatry (Mann 1999, Mann et al. 1999). Therefore, the distinction between demes of *S. pupula* certainly has a genetic basis, and differences among the demes may also be reflected in genes that have no immediate relevance to mating, for example, in nuclear-encoded rRNA genes.

In this study ITS and 18S rDNA sequences have been determined for a variety of clones and demes from *S. pupula* and *S. laevissima* to investigate the relationships between reproductive compatibility and genetic relationship. Great differences in ITS rDNA sequences were found to be correlated with sexual incompatibility. 18S sequence analyses of the studied clones and demes revealed the monophyletic origin of the genus *Sellaphora* and supported the close relationship of the genus with *Navicula pelliculosa* and the genus *Pinnularia*.

MATERIALS AND METHODS

Algal cultures. Eleven clones of *S. pupula* and one clone of *S. laevissima* were obtained from epipelic communities growing on mud at approximately 1 m depth in three Scottish lakes by D. G. Mann or V. A. Chepurnov (Table 1). Clones were isolated either by pipette or streaking on agar. In two cases (RECT-4 and RECT-5), the clones used here for molecular analysis were F1 clones reisolated from the

TABLE 1. The clones and demes (the names of *Sellaphora pupula* demes are those used informally at Edinburgh or referred to by Mann 1989, 1999, Mann et al. 1999) of *Sellaphora*, *Navicula pelliculosa*, *Pinnularia*, and *Lyrella* used in molecular genetic studies, their origins, voucher references, and GenBank sequence accession numbers.

Taxon and name	Deme	Clone identifier	Collection information	Voucher number	GenBank accession 18S/ITS
S. pupula RECT-1	Rectangular	BS13	Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 19 February 1997	E2997	—/AJ544660
S. pupula RECT-2	Rectangular	rect66	Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 12 February 1998	E3161	—/AJ544661
S. pupula RECT-3	Rectangular	BLA17	Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 23 March 1998	E3236	AJ544645/AJ544662
S. pupula RECT-4	Rectangular	(BS7)3	Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 19 February 1997; clonal F1 progeny reisolated by V. A. Chepurnov after intraclonal auxosporulation	E2996, E3259	AJ544646/AJ544663, AJ544664
S. pupula RECT-5	Rectangular	(BAL7)4	Balgavies Loch, near Forfar, Scotland, UK; coll. D. G. Mann, 14 February 1997; clonal F1 progeny reisolated by V. A. Chepurnov after intraclonal auxosporulation	E3004, E3267	—/AJ544665
S. pupula RECT-6	Rectangular	(BS14 × BM44)4	Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 19 February 1997; F1 clone of cross between clones BS14 and BM44, isolated by V. A. Chepurnov	E3253	—/AJ544666
S. pupula PSEUDOCAP-1	Pseudocapitate	18TmNE	Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann and V. A. Chepurnov, 21 September 1997	E3402	AJ544647/AJ544667–AJ544670
S. pupula PSEUDOCAP-2	Pseudocapitate	THR42	Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann, 23 March 1998	E3369	AJ544648/AJ544671
S. pupula PSEUDOCAP-3	Pseudocapitate	L845.4	Black Dog Lake Creek, Dakota County, Minnesota, USA; coll. D. B. Czarnecki, 19 June 1996 (L845); clonal reisolate by V. A. Chepurnov	E3345	AJ544649/AJ544672
S. pupula PSEUDOCAP-4	Pseudocapitate	(L845.5 × THR42)2	F1 clone of intercontinental cross between clones L845.5 (a clonal reisolate from L845) and THR42	E3384	AJ544650/AJ544673
S. pupula CAP	Capitate	BS50	Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 19 February 1997	E3220, E3221	AJ544651 (18S + ITS)
S. pupula SMALL	Small	8B	Blackford Pond, Edinburgh, Scotland, UK; coll. V. A. Chepurnov and D. G. Mann, 7 October 1996	E3201	AJ544652/AJ544677–AJ544679
S. pupula SM-BLCAP	Small blunt-capitate	6Tm	Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. V. A. Chepurnov and D. G. Mann, 6 October 1996; nonclonal, following at least one cycle of auxosporulation without reisolation	E3161	AJ544653/AJ544674, AJ544675
S. pupula BLUNT	Blunt	THR15	Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann, 23 March 1998	E3352	AJ544654/AJ544676
Sellaphora laevissima SCO	N/A	THR52	Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann, 23 March 1998	E3372	AJ544655/AJ544680
S. laevissima US	N/A	A79	West Lake Okoboji, Dickinson County, Iowa, USA; coll. D. B. Czarnecki, 25 August 1982	E3340	AJ544656 (18S + ITS)
Navicula pelliculosa	N/A	SAG 1050-3	New Haven Green, Connecticut, USA; coll. J. C. Lewin 1950	N/A	AJ544657/—
Pinnularia cf. *interrupta*	N/A	TE1	Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann, 19 January 1997	E3178	AJ544658/—
Lyrella atlantica	N/A		Port Chaligaig pier, Highland Region, Scotland, UK; coll. M. M. Bayer April 1999		AJ544659/—

Voucher material is held in the herbarium at the Royal Botanic Garden Edinburgh (E).

parental clones after intraclonal auxosporulation. SM-BLCAP was nonclonal, because it contained the F1 (and F2?) progeny of vigorous intraclonal auxosporulation within the original clonal culture (6Tm). The RECT-6 clone was an F1 clone derived from an interclonal cross. Two further clones of *Sellaphora* species (Table 1), representing North American isolates, were very kindly supplied by Dr. D. B. Czarnecki from the Freshwater Diatom Culture Collection at Loras College, Iowa, USA. PSEUDOCAP-4 was an F1 clone isolated from among the progeny of crosses between PSEUDOCAP-2 and PSEUDOCAP-3. Two clones of the *S. pupula* rectangular deme, BM44 and BS56, which were isolated from Blackford Pond at the same time as RECT-1 (Table 1), were used in mating experiments (because they were female clones, whereas most of the other clones studied were male) but not in the molecular genetic analyses. Details of these clones will be given in a forthcoming study on the mating system of the rectangular deme; voucher material is held at Edinburgh (accessions E3000 and E3001). Because almost all the clones studied were unisexual, it was impossible to maintain them beyond the current phase of size reduction: Intraclonal auxosporulation was absent or ineffectual, and the cells continued to divide mitotically and get smaller until they died. This is a major problem for long-term studies of pennate diatom species complexes.

Navicula pelliculosa (Bréb.) Hilse strain SAG 1050-3 was obtained from the "Sammlung von Algenkulturen" at the University of Göttingen, Germany. A *Pinnularia* species was isolated from the eastern subbasin of Threipmuir Reservoir and the marine *Lyrella atlantica* (A. Schmidt) D. G. Mann was obtained from west Scotland (Table 1). The identity of the *Pinnularia* clone cannot be determined with certainty because of inadequacies in current taxonomy. Clone TE1 valves closely resembled the valve illustrated in figure 190/3 by Krammer and Lange-Bertalot (1986), which they referred to as *P. interrupta* W. Smith. For molecular analysis, clones of *Sellaphora* species were grown in WC medium (Guillard and Lorenzen 1972) containing 0.005% Na_2SiO_3, in 50-mL Erlenmeyer flasks. *Navicula pelliculosa* was grown on diatom agar (Schlösser 1994). Cultures were kept at 16 or 18° C under a light–dark regime of 12:12-h or 10:14-h at a low light intensity of about 2–4 μmol photons $\cdot m^{-2} \cdot s^{-1}$ from white fluorescent bulbs. Stock cultures of the clones had previously been maintained as described by Mann et al. (1999). Because many of the *Sellaphora* demes are dioecious or strongly outbreeding and because of the obligate sexual phase in their life cycles, it is not possible to maintain most strains in long-term clonal culture. Voucher material comprising cleaned valves of each clone is therefore held in the diatom herbarium of the Royal Botanic Garden, Edinburgh (E) (Table 1).

Crossing experiments. Crosses were initiated by inoculating small aliquots of stock cultures of *Sellaphora* clones (in exponential growth phase) in pairs into 3–4 mL of fresh WC medium in the wells of 25-compartment Repli dishes. The mixed cultures were checked daily using an Axiovert inverted microscope (Zeiss, Oberkochen, Germany) for any sign of sexual activity.

DNA extraction, PCR, cloning, restriction digests, and sequencing. Cells of the 16 *Sellaphora* clones (Table 1) and *N. pelliculosa* were harvested by centrifugation (20 min at 4000 rpm), and the pellets were washed with extraction buffer (Friedl 1995). Cells were mechanically broken using a cell homogenizer (MiniBeadBeater, Biospec, Bartlesville, OK, USA) (Friedl 1995), and DNA was extracted using the Invisorb Plant Spin Kit (Invitek, Berlin, Germany), with extraction buffers as recommended by the manufacturer. PCR primer pair NS1 (Hamby et al. 1988) and ITS-4 (White et al. 1990) were used to amplify a rDNA region containing the 18S, ITS-1, 5.8S, and ITS-2 regions. The PCR protocol was as follows: 38 cycles of 94° C for 40 s, 50° C for 30 s, and 72° C for 60 s. All reactions were initiated with a 5-min denaturation at 95° C and ended with 7-min extension at 72° C. PCR products were cleaned with a High Pure PCR Purification Kit (Roche, Penzberg, Germany). 18S rDNA sequences for *Sellaphora* and *N. pelliculosa* were obtained directly from PCR products. For the ITS-1–5.8S–ITS-2 rDNA sequences, cloning of the PCR products was required, due to intraclonal sequence variation (see Results). For cloning, the pGEM-T Vector System I Kit (Promega, Madison, WI, USA) and DH5 cells of *Escherichia coli* were used. Plasmids were isolated using the Wizard Plus SV Miniprep DNA Purification System (Promega) and taken as template for sequencing reactions.

To investigate the sequence variation within a single clone (intraclonal sequence variation), 8–10 samples of plasmid DNA containing the ITS-1–5.8S–ITS-2 regions for the *S. pupula* clones RECT-1, PSEUDOCAP-1, SMALL, and SM-BLCAP were checked by restriction enzyme digests. Plasmid DNA was reamplified using the plasmid primers M13F and M13R, and the PCR products were digested (after purification) with restriction enzymes that recognize 4 nts-long motifs, viz. *Mbo* I (MBI Fermentas, St. Leon-Roth, Germany), *Hha* I (Promega, Madison, WI, USA), and *Bsh*1236I (MBI Fermentas). Digests were performed in 20-μL reactions that contained buffers as recommended by the manufacturer, 1 unit of enzyme, 0.2 μL BSA (if not included in the buffer), 1 μg of the purified PCR product, and sterile water. After an incubation for 3 h at 37° C and denaturing for 20 min at 65° C, an aliquot was run on a 2% agarose gel for 5 h at 60 V. For each different restriction pattern, one exemplar plasmid sample was sequenced.

Cycle sequencing over both strands was done with the Thermo Sequenase Sequencing kit with 7-deaza-dGTP (Amersham Pharmacia, Piscataway, NJ, USA). A set of nested primers (Elwood et al. 1985) and primers ITS2N and ITS3N (Beck et al. 1998) were used, which were CY5- or IRD-labeled and complementary to conserved regions of the 18S, 5.8S, and 26S rRNA coding regions. Plasmids were sequenced using CY5- or IRD-labeled M13 standard primers. Sequencing reactions were run on ALFexpress II (Amersham Pharmacia) and LI-COR L4200 automated sequencers (LI-COR, Lincoln, NE, USA).

Sequences of 18S rDNA for *Pinnularia* cf. *interrupta* and *Lyrella atlantica* were obtained by direct sequencing from PCR products. They form part of a separate study (unpublished data) but were added to the current data set to help reveal relationships among naviculoid taxa.

Alignment and phylogenetic analyses and ITS-2 secondary structure models. The rDNA sequences were manually aligned using BioEdit (http://www.mbio.ncsu.edu/RNaseP/info/programs/BIOEDIT/bioedit.html; Hall 1999). 18S rDNA sequences were compared with approximately 70 rRNA coding regions from other diatoms and heterokont algae. For 18S rDNA sequences, conserved rRNA secondary structures (Neefs et al. 1993) were used to refine the alignment. The final alignment was restricted to 18S rRNA coding regions from pennate diatoms and the sequences of the centric *Cymatosira belgica* Grun. (GenBank X85387) and *Papiliocellulus elegans* Hasle, von Stosch et Syvertsen (GenBank X85388). The latter two species were used as outgroup taxa, because in our initial analyses they appeared as a monophyletic lineage of centric diatoms that was most closely related to the pennates. The 18S rDNA reference sequences used for phylogeny reconstruction are listed in Table 2. Short sequences at either end of the 18S rRNA gene containing the PCR primer regions (positions 1–48 and 1765–1788 of the *Cylindrotheca closterium* (Ehrenb.) Lewin et Reimann sequence M87326) were excluded to adjust all sequences to equal lengths. With the 18S rDNA sequences, two more analyses

TABLE 2. Reference sequences from pennate diatoms used for the 18S rDNA phylogeny of Figure 3.

Species	GenBank accession
Amphora montana Krasske	AJ243061
Asterionellopsis glacialis (Castracane) Round	X77701
Bacillaria paxillifer (O.F. Müll.) Hendey	M87325
Cylindrotheca closterium (Ehrenb.) Reimann et Lewin	M87326
Eolimna minima (Grun.) Lange-Bertalot	AJ243063
Eolimna subminuscula (Manguin) Moser et al.	AJ243064
Fragilaria striatula Lyngb.	X77702
Gomphonema parvulum (Kütz.) Kütz.	AJ243062
Navicula cryptocephala Kütz.	AJ297724
Tryblionella apiculata Gregory	M87334
Phaeodactylum tricornutum Bohlin	AJ269501
Pseudo-nitzschia multiseries (Hasle) Hasle	U18241
Pseudo-nitzschia pungens (Grun. ex Cleve) Hasle	U18240
Rhaphoneis belgica Grun. in Van Heurck	X77703
Thalassionema nitzschioides (Grun.) Grun. ex Hust.	X77702

were done. In one additional analysis, the araphid pennates *Thalassionema* and *Fragilaria*, which appear more closely related to the raphids than are the centric diatoms in 18S rDNA analyses, were used as outgroup taxa to detect possible influences of the outgroup on resolution among the raphids and on the phylogenetic position of *Sellaphora*. An unrooted phylogeny of the full 18S rDNA sequences from *Sellaphora* demes only was calculated to test whether the resolution among demes and clones could be improved. A second data set was built from ITS-1–5.8S–ITS-2 rDNA sequences that represented all six demes of *S. pupula*; the intraclonal sequence variants found in clones of SMALL, SM-BLCAP, RECT-4, and PSEUDOCAP-1; and both clones of *S. laevissima*. Because of extensive variation in the primary structure and lengths of these sequences (Table 3), only a fraction of the sequence positions could be aligned unambiguously (see below). The sequence alignments and data matrices are available from T. Friedl and from Treebase (http://treebase.-bio.buffalo.edu/treebase/; 18S, accession no. M1533; ITS, accession no. M1534). Accession numbers for the 18S and ITS-1–5.8S–ITS-2 rDNA sequences newly determined in the current study are given in Table 1.

Three independent types of data analysis were used to assess the evolutionary relationships resolved in the rDNA phylogenies; all were implemented using PAUP* V4.0b10 (Swofford 2001). In maximum parsimony (MP) analyses, the sites were treated both unweighted and weighted (rescaled consistency index over an interval of 1–1000) (Bhattacharya and Medlin 1995). Introduced gaps were treated as missing data. Heuristic search conditions were with starting trees built stepwise with 10 random additions of taxa, using the tree bisection-reconnection branch-swapping algorithm to find the best tree. The best scoring trees were held at each step. For distance and maximum likelihood (ML) analyses, which are dependent on a particular specified model of nucleotide substitution, the program Modeltest, version 3.04 (Posada and Crandall 1998), was used to survey 56 possible models of DNA evolution to identify the model that best fit the three different rDNA data sets. For the 18S phylogenies as shown here, the TrN + I + G model (Tamura and Nei 1993) was chosen, with estimates of nucleotide frequencies A = 0.2554, C = 0.1831, G = 0.2718, T = 0.2897; a rate matrix with six different substitution types, assuming a heterogeneous rate of substitutions with a gamma distribution of variable sites (number of rate categories = 4, shape parameter $\alpha = 0.4846$); and the proportion of invariant sites that are unable to accept substitutions (pinvar) = 0.5988. For the ITS-1–5.8S–ITS-2 rDNA data set, the K80 + G model (Kimura 1980) was selected, with an equal distribution of nucleotide frequencies, a gamma distribution of variable sites (number of rate categories = 2, shape parameter = 0.3113), and a transition/transversion ratio of 1.5580. Distance trees were constructed using the minimum evolution (ME) criterion (Rzhetsky and Nei 1992) with the same heuristic search procedures as in the MP analyses. ML searches were done under the same model and settings as in the distance analysis with heuristic search conditions as in MP searches, except that the neighbor-joining method was used to build starting trees for branch-swapping. Bootstrap tests were done on MP and ME trees with 2000 replicates and on ML trees with 700 (18S data set) and 1000 (ITS data set) replicates.

Secondary structure models were constructed for ITS-2 in the RECT and PSEUDOCAP clones to check for CBCs (Gutell et al. 1994) among those clones whose sequences were rather similar and that might form a Z clade (see below). The sequences were submitted to *mfold version 3.1* (http://www.bioinfo.rpi.edu/applications/mfold/; Mathews et al. 1999, Zuker et al. 1999). Those predicted structures were selected that corresponded best to previously published ITS-2 secondary structure models that are conserved for chlorophytes as well as land plants (Mai and Coleman 1997).

TABLE 3. The demes and clones of *Sellaphora pupula/laevissima* from which the ITS-1–5.8S–ITS-2 rDNA regions were sequenced, grouped according to sequence similarity.

Clones and intraclonal sequence variants	Length of ITS-1 (bp)	Length of ITS-2 (bp)	Total of positions (incl. indels of 1–3 bp) different among clones / intraclonal sequence variants	Sequence differences within a group of clones or among intraclonal sequence variants
RECT-1,[a] -2, -3, -4a, -4b, -5, -6	358	310-318	23/5	92 positions and 3 indels of 4, 7, and 26 bp
PSEUDOCAP-1a,[b] -1b, -1c, 1d, -2, -3, -4	356	324-334	12/7	
SM-BLCAP-a, -b	261/255	313/303	n.a/18	92 positions and 2 indels of 35 and 5 bp
BLUNT	254	265	n.a.	
CAP	315	286	n.a.	
SMALL-a,[c] -b, -c	276/276/229	368/367/361	n.a./102	48 positions and 2 indels of 50 and 4 bp
laevissima-SCO, -US	312/355	390/379	92/n.a.	40 positions and 3 indels of 45, 4, and 4 bp

Only among RECT/PSEUDOCAP and SM-BLCAP/BLUNT clones, respectively, a plausible alignment of their ITS-1, -2 sequences was possible. Lengths of the ITS-1 and ITS-2 regions, sequence differences among clones and intraclonal sequence variants (where investigated, see text) are given. Intraclonal sequence variants are indicated by a small letter suffix added to the clone name.

[a]RECT-1 differed by a 7-bp insertion in ITS-2 from other RECT clones.

[b]PSEUDOCAP-1a–d differed by a 9-bp deletion in ITS-2 from other PSEUDOCAP clones.

[c]SMALL-a had two insertions of 50 and 4 bp compared with SMALL-c

RESULTS

Identities of Sellaphora demes and clones. Clones of *S. pupula* were provisionally classified into demes on the basis of their valve morphology alone, using our previous studies (Mann 1984, 1989a, 1999, 2001, Mann and Droop 1996, Mann et al. 1999) for guidance. This led to some errors. The identities of clones RECT-1 to -4 and RECT-6 (Table 1), all from Blackford Pond, were not controversial. All (Fig. 1, a–f) belong to the ''rectangular'' deme, which was originally recognized in Blackford Pond and has been described and illustrated in several studies (Mann 1989a, fig. 1, g–j, Mann 1999, fig. 52, Mann et al. 1999, figs. 4–6). Similarly, the CAP clone (Fig. 1, i and j) clearly belonged to the ''capitate'' deme from Blackford Pond (Mann 1989a, fig. 1, m–o, Mann 1999, fig. 53, Mann et al. 1999, figs. 1–3). Although they have very similar shapes, striation patterns, and striation densities (generally 19 or 20 in 10 μm) (Mann 1989a), the capitate and rectangular demes in Blackford Pond are clearly differentiated in size, capitate cells being consistently smaller and narrower than rectangular cells at equivalent stages in the size reduction cycle (Mann 1989a, fig. 2, a and b, Mann et al. 1999, figs. 7–9). For example, the RECT-4 valve shown in Figure 1d is shorter but nevertheless wider than the CAP valve in Figure 1j. The rectangular and capitate demes were already known (judging by valve morphology) to occur in other lakes besides Blackford Pond (Mann and Droop 1996, figs. 7, 8, 13, and 14, Taylor 1996) and RECT-5, derived from Balgavies Loch, a lake over 80 km distant from Blackford Pond, is clearly referable to the rectangular deme, not the capitate deme (Fig. 1, g and h; compare Fig. 1, a–f, contrast Fig. 1, i and j).

More problematic were the identities of the PSEUDOCAP clones (Table 1). We initially identified these clones through gestalt comparisons (i.e. by visual assessment of overall similarity) with other clones in culture at the same time, made via a Zeiss Axiovert inverted microscope (×40 dry lens). Accordingly, clone 18TmNE from Threipmuir Reservoir was initially assigned to the rectangular deme. Crossing data were apparently consistent with this identification (see below). By contrast, clones THR42, also from Threipmuir Reservoir but isolated 6 months later than 18TmNE, and L845, from central North America, were tentatively assigned to the capitate deme (as ''cf. capitate''), because they appeared slender, relative to

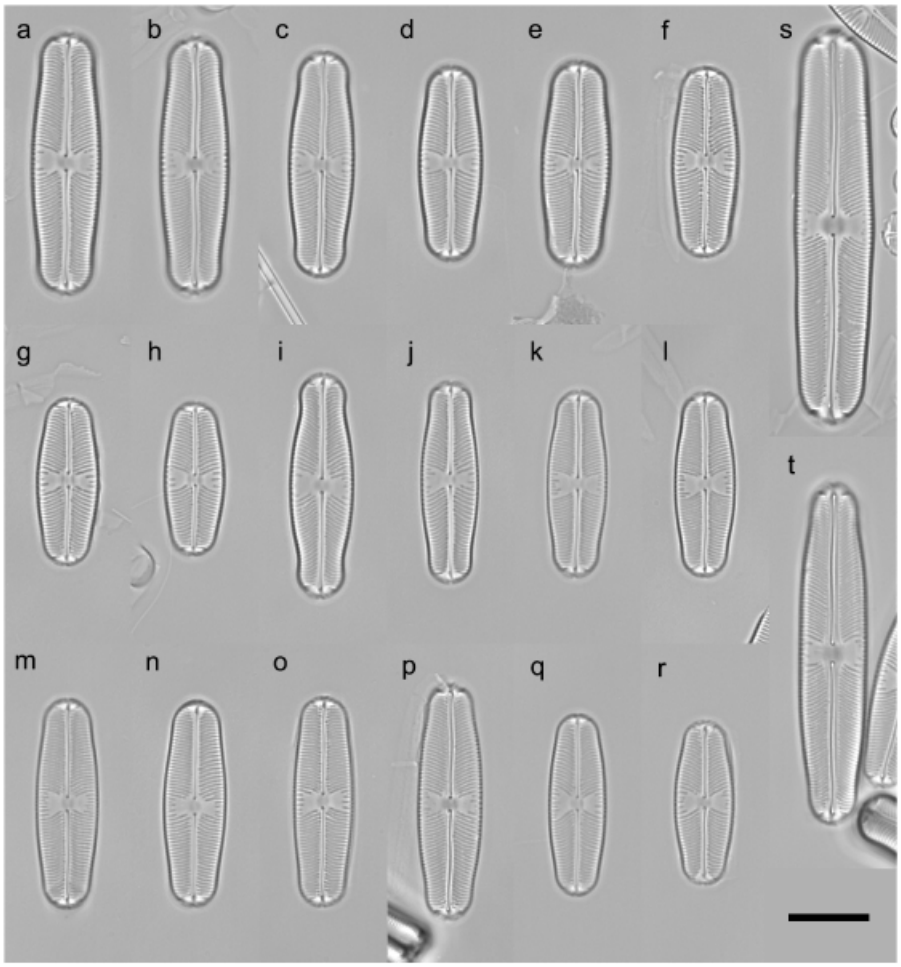

FIG. 1. Cleaned valves of *Sellaphora* clones, LM. Three demes are shown: rectangular (a–h, s), capitate (i, j), and the cryptic clade represented by the PSEUDOCAP clones (k–r, t). Scale bar, 10 μm. (a, b) RECT-1, Blackford Pond. (c, d) RECT-4, Blackford Pond. (e, f) RECT-2, Blackford Pond. (g, h) RECT-5, Balgavies Loch. (i, j) CAP, Blackford Pond. (k, l) PSEUDOCAP-1, Threipmuir Reservoir. (m–o) PSEUDOCAP-2, Threipmuir Reservoir. (p–r) PSEUDOCAP-3, Black Dog Lake Creek, Minnesota. (s) RECT-5, postauxospore valve. (t) PSEUDOCAP-3, postauxospore valve.

the rectangular clones in culture at the time (e.g. RECT-2, Fig. 1, e and f). It was not until after we had obtained the first molecular data for the clones that we were prompted to examine the morphology of the PSEUDOCAP clones in more detail, because clone 18TmNE had unexpectedly proved to have a very similar ITS and 18S rDNA sequence to THR42 and L845. By this time, the 18TmNE culture contained only very small cells, as a result of continued size reduction, but preserved valves from earlier phases of the size reduction cycle allowed comparisons with THR42 and L845 and also the RECT and CAP clones (Fig. 1). Close examination of valve morphology revealed great similarity between 18TmNE and THR42 in shape and size and stria pattern (Fig. 1, k–o) and a difference in stria density between these two clones and true capitate or true rectangular clones (i.e. CAP and RECT-1 to -6): 18TmNE and THR42 had 22–26 striae in 10 μm, whereas RECT-1 to -6 and CAP had 19–22 in 10 μm. We therefore refer to 18TmNE and THR42 as members of the "pseudocapitate" deme, labeling them PSEUDOCAP-1 and PSEUDOCAP-2. PSEUDOCAP-1 and PSEUDOCAP-2 are intermediate between true capitate and rectangular in their widths (Fig. 1). The American clone L845, from Minnesota, was similar to PSEUDOCAP-2 and proved to be compatible with it (see below), producing vigorous F1 offspring. We therefore refer to it here as PSEUDOCAP-3 and to an F1 clone of the PSEUDOCAP-2 × PSEUDOCAP-3 cross as PSEUDOCAP-4. In dimensions, shape, and striation pattern, PSEUDOCAP-3 was very similar to both PSEUDOCAP-1 and PSEUDOCAP-2 (Fig. 1, p–r), but it had a striation density (20–21 in 10 μm) closer to that of true capitate and rectangular clones (e.g. RECT-1 to -6 and CAP). A further difference between the PSEUDOCAP and RECT demes is that the valves of PSEUDOCAP are more delicate (note the difference in the thickness and intensity of the valve outline in Fig. 1, between a–h and k–r). The initial cells of PSEUDOCAP clones are narrower and shorter than those of RECT clones (Fig. 1, s and t).

Overall, the RECT, CAP, and PSEUDOCAP clones are all extremely similar (Fig. 1, a–r, Table 4), and it is doubtful whether any diatom taxonomist would have separated them, if there had been no previous studies of reproductive compatibility (Mann 1984, 1989a, 1999, Mann et al. 1999) and if the only data available were observations of cleaned valves. At some stages of the life cycle—such as in postauxospore cells (Fig. 1, s and t)—the demes are visibly distinct, but their identification becomes extremely difficult as cells approach their lower size limit (cf. Mann et al. 1999, figs. 15–35). The rectangular, capitate, and pseudocapitate demes must therefore be regarded as semicryptic. Ultrastructural differences between them are also only very subtle (unpublished data).

Three other *S. pupula* demes were included in the analysis, to provide context for the main focus, on rectangular, capitate, and pseudocapitate. The "small" deme in Blackford Pond was represented by clone 8B (*S. pupula* SMALL), which we have also used in mating experiments (unpublished data). The small deme, which has smaller and more delicate cells than the capitate or rectangular demes, also differs from them in valve outline and the shape of the central area (Table 4). It has already been well illustrated and characterized (Mann 1989a, table 1, fig. 1, p–r, Mann and Droop 1996, figs. 3, 9, and 15). The other two demes were morphologically similar to each other, both having narrow (generally <7.5 μm wide) linear-lanceolate valves (Table 4), and occurred together in Threipmuir Reservoir. The smaller celled of the two, which we have been calling "small blunt-capitate" (hence *S. pupula*

TABLE 4. Selected morphological characteristics of the demes of *Sellaphora pupula* analyzed.

	Rectangular	Capitate	Pseudocapitate	Small	Blunt	Small blunt-capitate
Valve length[a] (μm)	19–57	19–44	21–45	15–35	16–32	20–23
Valve width[a] (μm)	8.1–9.3	7.2–8.2	7.4–9.0	6.6–8	6.5–7.2	6.0–6.5
Overall shape	Linear-lanceolate	Linear-lanceolate	Linear-lanceolate	Lanceolate	Linear-lanceolate	Linear-lanceolate
Pole shape	Slightly rostrate–subcapitate	Subcapitate–capitate	Slightly rostrate–subcapitate	Rostrate	Slightly rostrate	Slightly rostrate–subcapitate
Stria density[b] (per 10 μm)	18–22	16–22	20–26	19–24	21–22	23–26
Stria pattern	Radial	Radial	Radial	Strongly radial	Slightly radial	Slightly radial
Polar bars	Parallel–radial	Parallel	Parallel–radial	Parallel	Parallel	Parallel
Central area	Irregular bow tie	Irregular bow tie	Irregular bow tie	Neat bow tie	Transversely elongate, ± rectangular	Transversely elongate, ± rectangular
Raphe course in LM	Sinuous	± Straight	± Sinuous	Straight	Straight	Straight
Robustness of valve[c]	+ + + +	+ + +	+ + +	+ +	+ +	+

Distinctions between the demes are subtle, and the ranges for any one character overlap considerably between demes.

[a]Length and width ranges are for natural populations (in culture, smaller cells are formed than occur in nature) and are complete for rectangular, capitate, and small only, where the full life cycle, from initial cells to gametangia, has been studied in detail. For pseudocapitate, the lower limit in nature has yet to be determined, and for this and small, blunt, and small blunt-capitate the length and width ranges are indicative only.

[b]Measured adjacent to the raphe system, excluding the central area.

[c]Visual estimate of the relative degree of silicification, on a four-point scale.

SM-BLCAP), was illustrated by Mann and Droop (1996, fig. 25); valves approximately 20 µm long are only 6–6.5 µm wide, with approximately 24 or more striae in 10 µm. The larger one ("blunt") is slightly more lanceolate. Here, valves that are approximately 20 µm long are approximately 7 µm wide, with approximately 21 striae in 10 µm; this deme is illustrated in the ADIAC database at http://rbg-web2.rbge.org.uk/ADIAC/db/adiacdb.htm. Further information on small blunt-capitate and blunt and other Threipmuir *S. pupula* demes will be given elsewhere, from analyses involving both recently developed and also longer established morphometric tools (du Buf and Bayer 2002).

Finally, we included two clones of a related *Sellaphora* species, *S. laevissima*, which came from different continents (Table 1) and differed slightly in their morphology. The American clone had wider valves (10 µm vs. 8.5 µm for valves approximately 35 µm long) and denser striation (17 vs. 15 in 10 µm) than the Scottish clone, but both fell within the limits for the species given by Krammer and Lange-Bertalot (1986).

Reproductive compatibility of Sellaphora *demes*. Previous observations (Mann 1984, 1989a, Mann et al. 1999) have shown, both in mixed seminatural populations (where natural populations are harvested and induced to become sexual en masse) and in experiments where clones are cultured together in pairs, that the Blackford rectangular, capitate, and small demes (which include clones RECT-1 to -4, RECT-6, CAP, and SMALL) are incompatible with each other. We also detected no mating between clones of any of these demes and either the SM-BLCAP clone or morphologically similar clones from Threipmuir Reservoir. In these incompatible combinations, no pairing took place (although there appears to be a residual attraction between cells in rectangular × capitate mixed cultures: Mann et al. 1999), and so the demes are reproductively isolated at the earliest prezygotic stages. The BLUNT clone was not used in mating experiments. Extensive observations of sexualized seminatural populations of mixed epipelic communities from Threipmuir Reservoir at frequent (approximately monthly) intervals over more than 2 years never revealed any case of interdemic compatibility involving the demes to which the PSEUDOCAP, BLUNT, and SM-BLCAP clones belong nor any case of interbreeding between *S. laevissima* and *S. pupula*.

Laboratory crosses were made to test compatibility within and between the rectangular and pseudocapitate demes. These revealed that many combinations of the RECT and PSEUDOCAP clones led to the formation of viable auxospores (Fig. 2). Exceptions were RECT-1 × RECT-2, RECT-1 × PSEUDOCAP-1, RECT-2 × PSEUDOCAP-1, and PSEUDOCAP-1 × PSEUDOCAP-2. Particularly significant results, in the light of subsequent molecular phylogenetic work (see below), are the successful matings between PSEUDOCAP-1 (from Threipmuir Reservoir), RECT-5 (from Balgavies Loch), and three clones (RECT-4, BM44, and BS56) from Blackford Pond (Fig. 2). Mating was not vigorous between PSEUDOCAP-1 and the Blackford clones RECT-4 or BS56 but was frequent with BM44. In all cases, cells paired and formed gametes, and healthy zygotes (auxospores) were formed after plasmogamy. PSEUDOCAP-1 behaved as a male clone, so that zygotes were formed in the cells of its partner (RECT-4, BM44, or BS56). We also observed mating between PSEUDOCAP-1 and two other clones from Blackford Pond, including the female 13B clone studied by Mann et al (1999). Mating between PSEUDOCAP-1 and the Balgavies clone RECT-5 was intense, and again PSEUDOCAP-1 cells behaved as males. Mating was also intense between RECT-5 and some Blackford clones, for example, RECT-1 and the parent clone of RECT-4, BS7. There was therefore no obvious distinction, on the grounds of compatibility, between RECT clones of different provenance (Blackford Pond and Balgavies Loch). However, we did not study the long-term viability of the F1 progeny of interprovenance crosses between RECT clones.

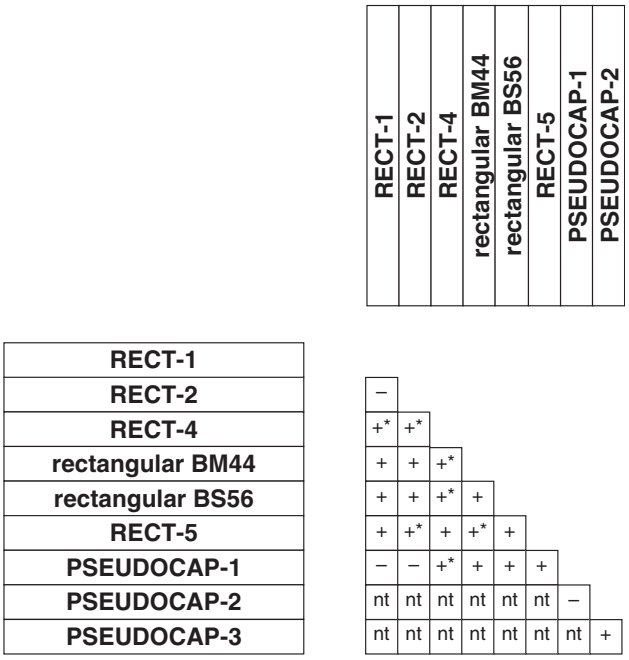

FIG. 2. *Sellaphora pupula*: reproductive compatibility between RECT, two additional clones of the rectangular deme from Blackford Pond (BM44 and BS56), and three PSEUDOCAP clones. Some crosses were not possible as a result of the loss of clones (through size reduction below the lower limit for viability or through accidental loss). In some cases, it was possible to substitute the F1 progeny of intraclonal (monoecious) auxosporulation; or a sister clone, belonging to the same F1 progeny of intraclonal auxosporulation of a single parental clone; or the parental clone itself. The crosses to which this applies are marked by an asterisk. nt, cross not made.

Clones PSEUDOCAP-2 and PSEUDOCAP-3 mated vigorously with each other and produced viable offspring, despite their origin on different continents; PSEUDOCAP-4 was isolated from among the F1

progeny. In crosses, all PSEUDOCAP-2 cells behaved as males and all PSEUDOCAP-3 cells as females. Unfortunately, by the time we discovered from molecular data that PSEUDOCAP-1 (which usually behaved as a male in crosses with RECT clones) was closely related to PSEUDOCAP-2 and -3, it was close to the lower size limit of the life cycle and was so moribund that further mating studies were impossible. Further mating tests involving the male PSEUDOCAP-2 were prevented by the loss of all female RECT clones. The American clone PSEUDOCAP-3 was capable of intraclonal auxosporulation (as well as outcrossing with PSEUDOCAP-2), whereas the Scottish PSEUDOCAP-2 apparently was not. Variation in the intensity of intraclonal auxosporulation has also been found among RECT clones (unpublished data).

Phylogenetic position of Sellaphora *within the diatoms*. Full 18S rDNA sequences were determined for 14 clones, comprising representatives of six demes of *S. pupula*, the two clones of *S. laevissima*, and *N. pelliculosa* (Table 1). The sequences of *Pinnularia* cf. *interrupta* and *Lyrella atlantica* were added to the data set. Among the four clones of PSEUDOCAP, there were differences at only two positions and the sequence of just one clone (PSEUDOCAP-4) was used in the phylogenetic analyses. No sequence difference was found between the two clones of RECT from which the 18S rDNA was sequenced, and therefore just one (RECT-3) was used for tree construction (Fig. 3). The alignment used for phylogeny reconstruction was 1698 positions long and contained 415 variable positions, of which 250 were parsimony informative. Among the *Sellaphora* clones studied, there were 49 variable sequence positions in the 18S rDNA, of which 31 were parsimony informative. The shortest genetic distances in the tree (Fig. 3) were between the PSEUDOCAP and RECT demes and between the two clones of *S. laevissima* (0.0018, corresponding to a difference of only three positions in the full 18S rDNA sequence). Most of the sequence variations among the *Sellaphora* clones were restricted to the V1 and V2 regions in the 18S rRNA secondary structure model of Neefs et al. (1993).

All the *Sellaphora* clones together formed a single lineage whose monophyletic origin was well supported in bootstrap tests, except in the ML analysis (Fig. 3). Relationships among the *Sellaphora* clones were not unambiguously resolved, however, because of the low number of informative sites. Only two features were well supported in bootstrap tests: a clade comprising clones RECT-3 and PSEUDOCAP-4 and the sister-group relationship of this clade with CAP (Fig. 3). The position of *S. laevissima*, though a distinct species in current classifications (Krammer and Lange-Bertalot 1986), was ambiguous. *Sellaphora laevissima* was a sister group to all clones of *S. pupula* only in the ML and MP analyses, but this relationship did not receive bootstrap support ($<50\%$). Both clones of *S. laevissima* were sister to *S. pupula* BLUNT in ME distance analysis, but this received insignificant support in bootstrap tests (62%). In an additional analysis where the araphids *Thalassionema* and *Fragilaria* were used as outgroup taxa, no better resolution could be achieved among the *Sellaphora* clones. However, in an unrooted 18S rDNA phylogeny of *Sellaphora* sequences alone, without excluding any sequence positions, a sister-group relationship of *S. pupula* BLUNT with both *S. laevissima* clones was well supported in bootstrap tests (tree not shown).

A sister-group relationship of the *Sellaphora* lineage with *Eolimna minima* was well supported in bootstrap tests, except in the ML analysis (Fig. 3). The clade most closely related to the *Sellaphora–Eolimna* clade was apparently a well-supported clade containing *N. pelliculosa* and *P.* cf. *interrupta*; this link received high (MP, ML) or only limited (ME) bootstrap support. The clade containing *Sellaphora*, *E. minima*, *N. pelliculosa*, and *P.* cf. *interrupta* is referred to here as the suborder Sellaphorineae, following Round et al. (1990, pp. 128, 657) (Fig. 3). *Amphora montana* and *Eolimna subminuscula* appeared to be the closest relatives of the Sellaphorineae among the other taxa sampled. However, there was little or no bootstrap support for any of the nodes between *Phaeodactylum* and *N. pelliculosa*, so that the exact positions of the *Gomphonema parvulum–Lyrella atlantica* clade, *Phaeodactylum tricornutum*, and *Navicula cryptocephala* within the raphid pennate diatoms could not be determined. The two genera, *Eolimna* and *Navicula*, are apparently not monophyletic (Fig. 3), and the monophyletic origin of the order Naviculales received only moderate (ML, MP) or no bootstrap (ME) support (Fig. 3); this clade even contained three species belonging (in the classification of Round et al. 1990) to other orders, viz. *Amphora montana* (Thalassiophysales), *Lyrella atlantica* (Lyrellales), and *Gomphonema parvulum* (Cymbellales). A monophyletic origin of the Bacillariales was moderately supported (Fig. 3). When the araphids *Thalassionema* and *Fragilaria* were used as alternative outgroup taxa, no better resolution was achieved.

ITS rDNA sequence variation within Sellaphora. To investigate relationships among clones of *S. pupula/laevissima*, the ITS-1–5.8S–ITS-2 rDNA regions were sequenced. The lengths of the ITS-1 + 2 regions differed considerably, between 519 (*S. pupula* BLUNT) and 734 bp (*S. laevissima* US, Table 3). Sequence variation in the ITS rDNAs among the *Sellaphora* demes was so high that an unambiguous alignment over the whole lengths of these regions was not possible. Based on the difficulty of producing plausible alignments, five groups of sequences could be distinguished. One group consisted of the *S. pupula* RECT and PSEUDOCAP demes, a second of SM-BLCAP and BLUNT, and three more groups were formed by CAP, SMALL, and the *S. laevissima* clones (Table 3). Within a group, the ITS-1 and -2 sequences were readily aligned. Length differences among clones within a group were mainly due to indels of 4–35 bp in the ITS-2 region; *laevissima*-US, however, had an insertion of 45 bp in ITS-1 compared with *laevissima*-SCO (Table 3).

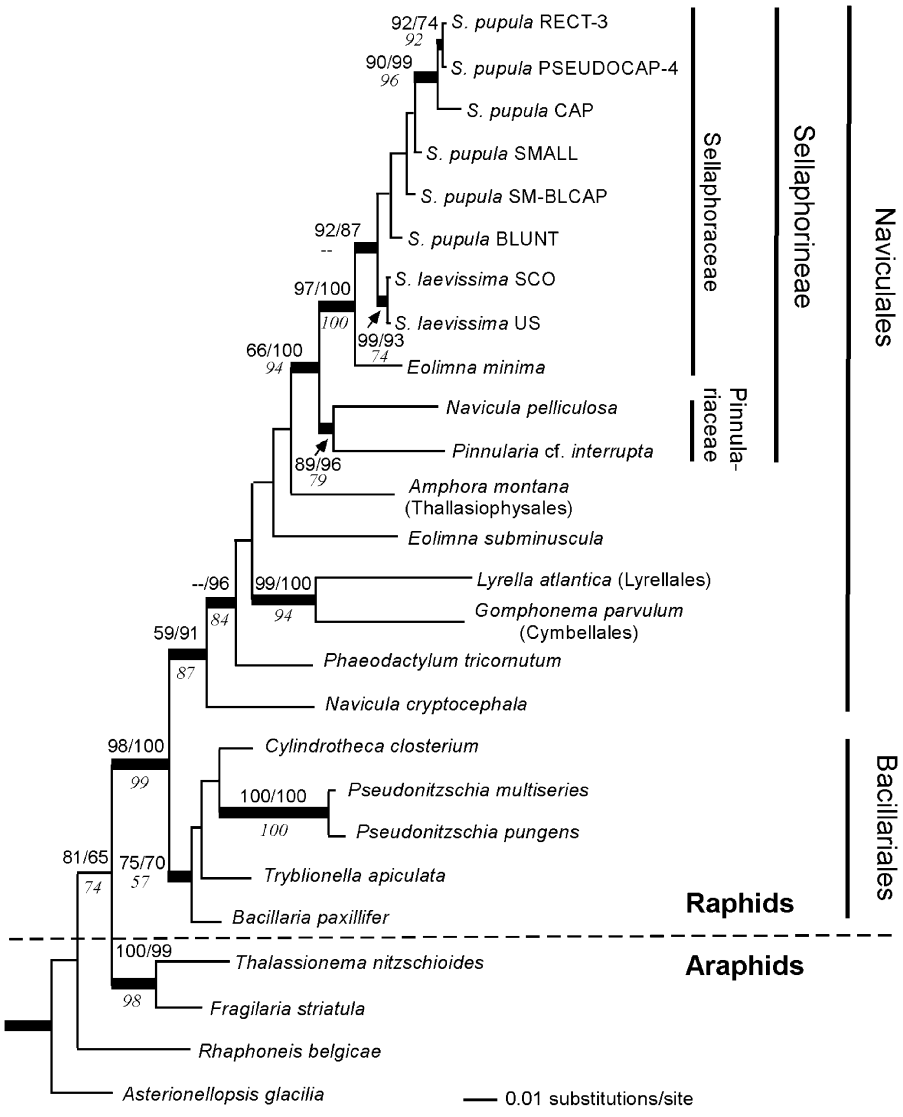

FIG. 3. Maximum likelihood phylogeny of 18S rDNAs from *Sellaphora*, *Navicula pelliculosa*, *Pinnularia* cf. *interrupta*, *Lyrella atlantica*, and other pennate diatoms. Thick lines mark internal nodes that were resolved in all distance, maximum parsimony, and maximum likelihood trees (see text). Values above internal nodes are bootstrap values from 2000 replications as calculated from minimum evolution/weighted parsimony; values below branches were calculated from maximum likelihood with 700 replications. Only bootstrap values above 50% are shown. The outgroup taxa (centric diatoms, see text) were pruned away from the tree.

Before phylogenetic analyses, we investigated whether there is significant variation in the ITS-1–5.8S–ITS-2 sequence within single clones of *S. pupula*. The RECT-4, PSEUDOCAP-1, SMALL, and SM-BLCAP clones were selected as examples. For each of these clones, 8–10 samples of plasmid DNA containing the ITS-1–5.8S–ITS-2 regions were checked by restriction enzyme digests. In each of the four clones, differences in the restriction patterns were found that indicated that each clone possesses several intraclonal sequence variants. For each different restriction pattern, one exemplar plasmid sample was sequenced and considerable sequence variation was found (Table 3). Within SMALL, three types of ITS-1–5.8S–ITS-2 sequences were detected (SMALL-a, -b, -c), which varied at 48 positions. In addition, the SMALL-a variant had two insertions, 50 bp and 4 bp in length, compared with the other two variants (Table 3). Within SM-BLCAP, two types of sequences were found, which differed at 18 sequence positions (Table 3). Within PSEUDOCAP-1, four types of ITS-1–5.8S–ITS-2 sequences were found that varied at seven positions (Table 3). Within RECT-4, two types of sequences were found that differed at five positions (Table 3).

Interclonal and interdemic relationships within Sellaphora. To test whether the two demes with rather similar ITS-1–5.8S–ITS-2 sequences and intraclonal sequence variation, RECT and PSEUDOCAP, are distinct from each other, an unrooted ML tree of the full PSEUDOCAP and RECT clone sequences (which were readily aligned) was constructed. The four ITS sequence variants of PSEUDOCAP-1 formed a single lineage within a clade representing PSEUDOCAP (with no bootstrap support, however) and the seven RECT sequences formed another clade (tree not shown). Therefore, only one pair of sequences from each deme, PSEUDOCAP-1a and PSEUDOCAP-4 and RECT-4a and RECT-5, was selected for investigating phylogenetic relationships among all *Sellaphora*. Only 156 sequence positions

(70/55 variable/parsimony informative sites) from the ITS-1 and ITS-2 rDNAs could be aligned with confidence and used for phylogenetic analysis (for details, see the data set, accession no. M1534 available from TREEBASE, http://treebase.bio.buffalo.edu/treebase/). The 5.8S rDNAs (155 sequence positions) provided another 16/10 variable/informative positions. The phylogenetic analysis showed the same division of the demes (Fig. 4) as had been found by inspection of the ITS sequences and their alignment. The RECT and PSEUDOCAP sequences together formed a single clade, which was well supported in bootstrap tests. The RECT–PSEUDOCAP clade was sister to CAP, and this relationship too was well supported (Fig. 4). A monophyletic origin of the BLUNT and SM-BLCAP clones was highly supported, as was the single origin of both *S. laevissima* clones (Fig. 4). A sister-group relationship of SMALL to the SM-BLCAP–BLUNT clade was found in all analyses but was not supported in bootstrap tests (Fig. 4). The *S. laevissima* lineage was sister to the *S. pupula* SMALL–BLUNT–SM-BLCAP clade, but our tree is unrooted and so it is unclear whether or not *S. pupula* is monophyletic or paraphyletic with respect to *S. laevissima*.

ITS-2 secondary structure models. In view of the similarity between the RECT and PSEUDOCAP sequences and because of the availability of breeding data, secondary structure models were constructed for ITS-2 in RECT and PSEUDOCAP and checked for CBCs (Fig. 5). The predicted consensus secondary-structure model suggested the presence of three helices (I, II, III). Though the obtained structures were all very similar to each other, a few stem- and loop-regions with small variations were noted among the clones (Fig. 5). A total of 20 positions and four indels 2–17 nts long were found in which all PSEUDOCAP clones differed from all RECT clones (Fig. 5). Among them were only four one-sided CBCs, and these were located in the stem region of helices I and II.

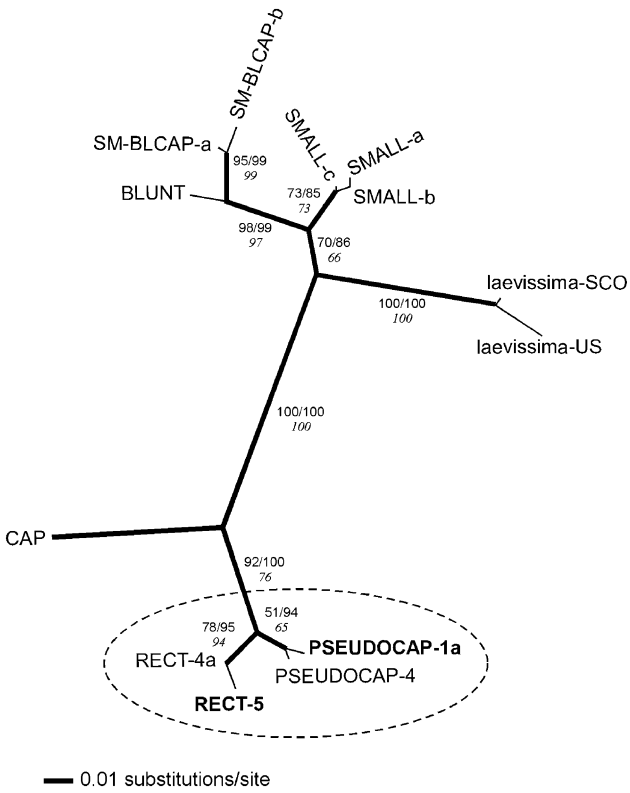

FIG. 4. Unrooted maximum likelihood phylogenies of ITS-1–5.8S–ITS-2 rDNA sequences from clones and intraclonal sequence variants of *Sellaphora*. Thick lines mark internal nodes that were resolved in all distance, maximum parsimony, and maximum likelihood trees (see text). Values above are bootstrap values from 2000 replications as calculated from minimum evolution (left), weighted maximum parsimony (right), or from 1000 replications as calculated from maximum likelihood. Only bootstrap values above 50% are shown. Dashed circle marks putative Z clade. Clones that produced a viable F1 generation are in bold.

DISCUSSION

Interdemic relationships and sexual compatibility within Sellaphora pupula. Interbreeding was observed among rectangular and pseudocapitate clones, but no sexual reproduction was observed in RECT-1 × RECT-2, RECT-1 × PSEUDOCAP-1, RECT-2 × PSEUDOCAP-1, and PSEUDOCAP-1 × PSEUDOCAP-2. Attempts to cross clones can be unsuccessful for any of several reasons: 1) the clones may belong to different reproductively isolated demes (see above), 2) one or both clones may be outside the sexual size range, 3) one or both clones may not be in appropriate physiological condition (sexual reproduction in compatible *Sellaphora* is most vigorous when cells are in exponential growth phase), or 4) the clones may be unable to mate because they have the same mating type. All our crossing experiments involved clones that were within the size range for sexual reproduction (as evidenced by positive results in several pair-wise combinations of clones) and were always initiated from vigorously growing stocks, so that 2) and 3) can be ruled out. Possibility 1) is negated by the pattern of successful mating that shows no clear-cut reproductive barrier between the rectangular and pseudocapitate demes. Thus, the four cases of incompatibility among RECT and PSEUDOCAP clones probably reflect the mating types of the clones being tested. This is almost certainly true for RECT-2 × PSEUDOCAP-1 because, in crosses with other clones (e.g. BM44 and BS56), RECT-2 and PSEUDOCAP-1 always behaved as males, implying that they are of the same mating type; the same applies to PSEUDOCAP-1 and PSEUDOCAP-2. However, the mating system is clearly not a simple bipolar type, because although RECT-1 and RECT-2 were unable to mate with each other and could both mate with RECT-4, all three could mate with BM44 and BS56. Previous studies of the rectangular deme in Blackford Pond have also indicated that it may have a complex mating system (unpublished observations), but even so, some combinations of clones produce few or no auxospores. For example, rectangular clone 13B, one of the clones

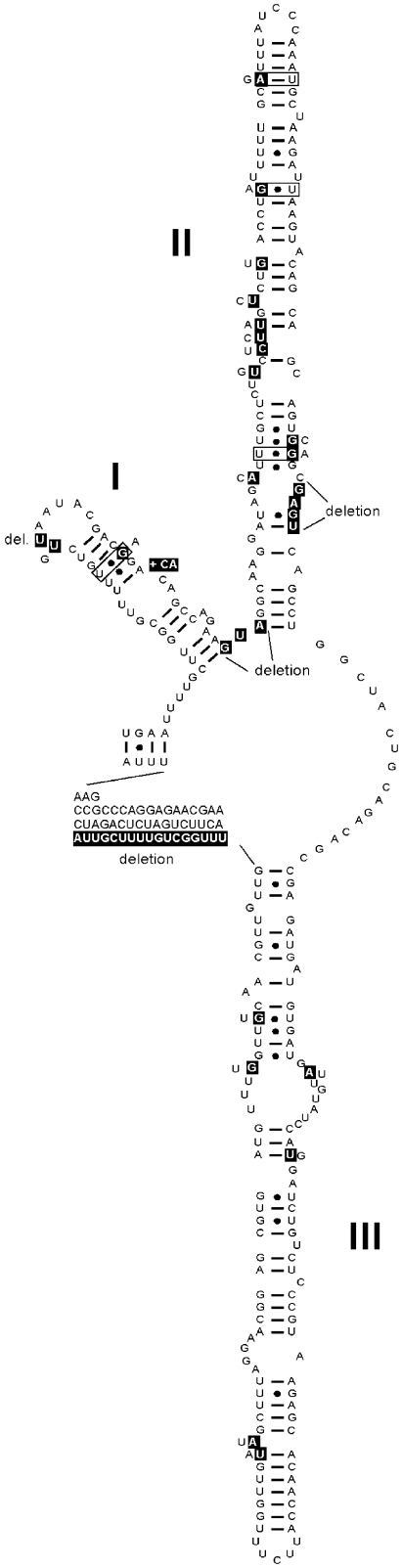

FIG. 5. ITS-2 secondary structure of the *Sellaphora pupula* clone PSEUDOCAP-1. Sequence positions in which all PSEUDOCAP clones differed from all RECT clones are indicated by a white letter on black background and the corresponding base of the RECT-5 sequence is shown next to the sequence position. One-sided compensating base changes are boxed.

used by Mann et al. (1999), did not mate with BM44, because both were apparently of the same mating type: cells of both always acted as females in compatible crosses with other clones (i.e. their gametangia always produced passive gametes during sexual reproduction) (Mann 1989b, Mann et al. 1999). Other clones were able to mate with both 13B and BM44. By contrast, the capitate deme exhibits an apparently simple mating system, with a clear differentiation into male and female clones; successful auxosporulation is possible only when both are present (Mann et al. 1999).

The division of the 16 clones of *S. pupula* and *S. laevissima* into groups, based on similarities in their ITS sequences and on phylogenetic analyses of the ITS-1, -2, and 18S sequence data sets, is congruent with groupings based on sexual compatibility: There is no case where molecular data indicate a closer relationship than is implied by mating data. The most closely related demes, according to the sequence data, are rectangular and pseudocapitate (the RECT and PSEUDOCAP clones), with the capitate deme somewhat less closely related. The degree of ITS sequence variation corresponds directly to the capacity for interbreeding. The RECT-1 to -4 and RECT-6 clones, all from Blackford Pond, and the RECT-5 clones from Balgavies Loch were all found to be capable of mating and producing a viable F1, either directly or via other rectangular deme clones (e.g. BM44, BS56), if their mating types made direct interaction impossible; indeed, RECT-6 is an F1 clone produced by crossing two other Blackford clones, the female clone BM44 and the largely male clone BS14. The ITS rDNA sequences of RECT-1 to -6 are identical except at 23 positions. The PSEUDOCAP clones form a group that is clearly distinct from the RECT clones in ITS sequence, but the two demes are not isolated by intrinsic reproductive isolation mechanisms, at least until the F1. However, the considerable sequence divergence between the rectangular and pseudocapitate demes in apparently "neutral" parts of the genome suggests that, in nature, there must be restricted or no gene flow between the demes. One possibility is that the demes are ecologically separated: Blackford Pond and Threipmuir Reservoir have very different epipelic diatom communities (unpublished observations), and Blackford Pond is a hypertrophic parkland pond (elevation approximately 70 m) within the city of Edinburgh, surrounded by houses and horticultural allotments, whereas Threipmuir is a peaty lake at moderate elevation (250 m) within a moorland catchment. Hence, it is possible that the rectangular and pseudocapitate demes do not meet in nature and their ITS sequences might be expected to diverge. Alternatively, there may be postzygotic reproductive isolation that we did not detect because we did not examine formation of an F2; in the *Achnanthes brevipes* species complex we demonstrated that two morphological variants, from different habitats, are compatible and form a vigorous F1, but this is unable to produce an F2 or backcross with parental strains (Mann 1999, p. 472).

Or reproductive isolation could be maintained by a combination of ecological and F1 breakdown.

The position of the clones PSEUDOCAP-2 to -4 within the same clade as PSEUDOCAP-1 and RECT clones and the relatively high sequence variation between the PSEUDOCAP clones and CAP were unexpected, because our preliminary identification, based on visual inspection of cell shape and size, had been that the PSEUDOCAP-2 and -3 clones and the CAP clone probably belonged to the same deme. Sequence analyses, however, reveal unambiguously that the PSEUDOCAP clones belong together and that their nearest relatives among the clones studied are the RECT clones. Careful studies of morphology show that the PSEUDOCAP clones can be distinguished morphologically from either CAP or RECT clones. However, the differences are very subtle, and separation is made more difficult by variation (e.g. in stria density) within each deme. To make further progress, both in establishing the distributions and ecology of the pseudocapitate and rectangular demes and in determining the extent of gene flow between them, it will be essential to find reliable ways to identify them in natural populations, using sensitive morphometric methods or molecular probes.

The capitate deme (represented by CAP) cannot interbreed with the rectangular deme (Mann 1989a, Mann et al. 1999), and the CAP ITS sequence is so distinct from those of the RECT and PSEUDOCAP clones that it is not possible to produce a full unambiguous alignment. However, the 18S and the ITS data sets, and also an unpublished *rbc*L data set, all show that the capitate deme is the nearest relative of the rectangular–pseudocapitate deme complex (RECT and PSEUDOCAP). Correlated with this, when male rectangular clones are mixed with female capitate clones or female rectangular with male capitate, there is a reciprocal stimulation of activity, but bonding between cells does not occur. More distantly related demes, such as the SMALL deme, appear not to show such residual sexual interactions with rectangular or capitate cells, either in seminatural populations or in mixed culture. However, not every pair-wise combination has been checked.

The ITS sequences of the two *S. laevissima* clones, from Europe and North America, are mostly alignable, but there are many significant differences between them, in base substitutions and indels. We have no crossing data for these clones, but the extent of divergence in ITS sequence agrees with preliminary morphological analyses (unpublished observations: the two clones differ in quantitative valve characters) that suggest the concept of *S. laevissima* adopted, for example by Krammer and Lange-Bertalot (1986), is too broad. *Sellaphora laevissima*, like *S. pupula*, is a species complex that should probably be split into several or many separate species. The 18S analysis places *S. laevissima* as sister taxon to the *S. pupula* complex, but there is no support from bootstrap tests for this. The ITS rDNA tree is unrooted, and so it is impossible to say whether or not it supports monophyly of *S. pupula*. It is possible that *S. laevissima* has evolved from within the *pupula* complex, despite the absence of polar bars in *laevissima*. VanLandingham (1964) recorded *S. laevissima* (as *Navicula wittrockii* f. *fusticulus*) from Miocene diatomites in Washington, USA, and *S. pupula* is also known from Miocene deposits (Glezer et al. 1974), which sets a minimum age for the separation of at least some *laevissima* and *pupula* lineages and indicates that the *S. pupula* and *laevissima* complexes have been diversifying for well over 5 million years.

Does morphology reflect genetic and reproductive relationships in the Sellaphora pupula *complex*?. A full assessment of congruence between different data sets—morphological, molecular, and reproductive—must wait until all possible combinations of demes have been tested for compatibility in culture. The only demes for which we have ± comprehensive data are rectangular, capitate, pseudocapitate, and small. Molecular and mating data agree that the rectangular and pseudocapitate demes are very closely related to each other and somewhat less closely related to capitate. This agrees reasonably well with morphology. However, molecular data cluster the blunt and small blunt-capitate demes, which have linear-lanceolate valves like the rectangular–capitate–pseudocapitate clade, with the small deme, which has lanceolate valves and markedly rostrate apices (Mann 1989a, Fig. 1, p–r, Mann and Droop 1996, Figs. 3, 9, and 15). In this case, gestalt assessment of morphological similarity (a cladistic analysis of morphology is impossible, because characters are quantitative and continuous, and the ranges exhibited by different demes overlap) does not suggest the same relationships as molecular sequence data. Our *rbc*L and 18S rDNA data on other *S. pupula* demes (unpublished data) confirm that the relationship between morphological similarity and genetic relationship is not tight.

The relatively wide evolutionary separation between the capitate and rectangular demes that is implied by their markedly different and partly unalignable ITS sequences is not something that would have been predicted from morphology. In our experience, even trained diatomists usually have difficulty in correctly discriminating between capitate and rectangular (and other linear-lanceolate demes in the same size range) and regard them as essentially "the same," yet clearly these are distinct biological species. Overall, then, morphology alone may be a poor guide both to species (and infraspecific) boundaries and interspecies relationships in the *S. pupula* complex and probably in other diatoms also, at or near the species level.

Z and CBC clades. A correlation between sexual compatibility and sequence differences in the ITS-2 rDNA region has been found for colonial green algae, the Volvocales, and later for *Chlamydomonas reinhardtii* (Coleman et al. 1994, Coleman and Mai 1997). These findings led to the hypothesis that organisms capable of interbreeding and producing viable zygotes (Z

clades) will be much more uniform in their ITS rDNAs, compared to the variation between organisms that cannot interbreed (Coleman et al. 1994): Members of a Z clade will represent just one ''ITS type,'' which is defined as a group of organisms whose ITS sequences vary by no more than about 10% (Coleman et al. 1994). The *Sellaphora* data are clearly consistent with the idea that Z clades exhibit less intraclade than interclade variation in ITS, and the maximum variation found within the RECT + PSEUDOCAP Z clade was only 7.3% (between RECT-4b and PSEUDOCAP-1d). The Volvocales contain a conserved region of 116 bp in the ITS-2, and algal strains capable of interbreeding show essentially no sequence variation in this region. We were unable to identify an analogous region in the ITS-2 of *Sellaphora*.

A group of organisms where there are no CBCs (Gutell et al. 1994) was termed a CBC clade by Coleman (2000), who suggested that if two organisms differ with respect to a two-sided CBC in their ITS-2, no gamete interaction will occur between them, that is, ITS-2 CBC clades will equate to Z clades. This hypothesis is supported by investigations of only a few algal groups, viz. *Closterium ehrenbergii* (Ichimura and Kasai 1996) and Volvocales (Coleman 2000, Denboh and Ichimura in Coleman 2000). We constructed putative ITS-2 secondary structure models for all the RECT and PSEUDOCAP clones, and the only variations found in stem regions were one-sided CBCs (U–A $\leftrightarrow$ U $\cdot$ G). Therefore, the RECT and PSEUDOCAP clones may be regarded as belonging to the same CBC clade. Although reproductive compatibility for those clones sequenced here has only been shown for RECT-5 $\times$ PSEUDOCAP-1, this finding may indicate that there is an equivalence of CBC and Z clades in the rectangular and pseudocapitate demes of *S. pupula*, as hypothesized by Coleman (2000) for chlorophytes. By contrast, two-sided CBCs (C–G $\leftrightarrow$ A–U) have been detected in helix 21-1 of 18S rRNA (Mann 1999, fig. 44), between Blackford clones of the rectangular and capitate demes (although the clones used differed from those studied in the present paper), correlating with apparently complete prezygotic reproductive isolation. To test further the equivalence of CBC and Z clades in *S. pupula*, it will be necessary to create reliable ITS-2 secondary structure models for the other demes. Because the ITS sequences among other demes showed a very high degree of variation, we predict several CBCs here. It will be challenging and interesting to test Coleman's hypothesis more widely in diatoms and in other algal groups where crossing experiments can be performed to examine whether CBCs can be used as a marker that two organisms have reached the point in evolution where their gametes are no longer able to interact.

Intraclonal ITS variation. Comparisons of ITS-1 and ITS-2 sequences in four of the *S. pupula* clones revealed that intraclonal, presumably intraindividual, variation in ITS is present in the *S. pupula* species complex. The degree of variation varied but was relatively low: There was a maximum of 48 bp sequence variation (5.9%) and in one case a deletion of a 50-bp section. A sequence divergence of 5.9% is less than that found within a single multiclone Z clade, for example, RECT + PSEUDOCAP (7.3%). When different paralogues of a clone were included in the phylogenetic analyses, we found the sequence variations were too minor to affect the phylogenetic relationships of the clones in the ITS phylogeny. Therefore, the ITS-1, -2 region can still be regarded as a useful tool to investigate the phylogenetic relationships within the *S. pupula* species complex. Similar findings were made by Deduangboripant and Cronk (2000), who found intraindividual variation of up to 5% in ITS sequences in the angiosperm genus *Aeschynanthus*; here too intraindividual variation did not affect phylogenetic reconstruction. In *Sellaphora*, the 5.8S rDNA was also found to exhibit intraclonal variation, at 1–5 positions. 18S rDNA was not checked for intraclonal variation in the present work. However, in two clones, CAP and SM-BLCAP, there were one or two positions where the primary structure could not be unambiguously resolved, which may reflect the presence of different paralogues. Intragenomic variation, even in the coding regions of the rDNA, has been detected in several very different organisms, such as angiosperms (Scrophulariaceae) (Ritland 1993) and flies (Diptera) (Tang 1996). In the mammal *Misgurnus fossilis*, two different types of 5.8S rDNAs were found, one characteristic of somatic cells and the other of oocytes (Mashkova et al. 1981). Whether the different ITS paralogues of *S. pupula* also have such a functional cause is unknown, and it will be interesting to check other diatom species for similar intragenomic variation.

Phylogenetic position of the genus Sellaphora. Phylogenetic analysis of the 18S rDNA confirmed, as expected, that *Sellaphora* is a member of the ''raphids,'' that is, pennate diatoms with a raphe. In addition, different *S. pupula* demes and *S. laevissima* have a single origin, which suggests that *Sellaphora*, as presently defined, is a monophyletic genus. This conclusion is also supported by *rbc*L and other 18S rDNA analyses (unpublished observations). However, *Eolimna* and *Navicula*, which provided the closest relatives of *Sellaphora* among the species included in our analysis, appear to have several origins. *Eolimna minima* was found within a well-supported clade with the *Sellaphora* demes and could therefore be regarded as belonging to the Sellaphoraceae, or even to *Sellaphora* itself. *Navicula pelliculosa* is closely related to both the Sellaphoraceae and *P.* cf. *interrupta*, whereas *N. cryptocephala* and *E. subminuscula* are phylogenetically distant from the Sellaphorineae. The position of *N. pelliculosa* within the Sellaphorineae clade is consistent within the suggestion of Round et al. (1990, p. 65) that this species is related to *Sellaphora*, but the relationship appears to be more distant than Round et al. thought. Round et al. did

not give reasons for their opinion, but *N. pelliculosa* sensu Reimann et al. (1966) has uniseriate striae containing simple round poroids (contrast the elongate poroids of *Navicula* sensu stricto), central raphe endings that are deflected toward the primary side internally (and externally), and a small but distinct central area; all these are characteristics also present in *Sellaphora*. *Navicula pelliculosa* lacks the complex internal chambers (alveolate striae) present in *Pinnularia* (cf. Round et al. 1990, p. 556). The micrographs of Reimann et al. (1966) and Chiappino and Volcani (1977) do not include sections of interphase cells, but the pre- and postcytokinetic cells they illustrate show chloroplast positions and structure that agree well with what is known for *Sellaphora* (Mann 1985, 1989b).

The robust though apparently distant relationship (judging by 18S rDNA branch lengths) between the naviculoid *Lyrella* and the heteropolar genus *Gomphonema* links two orders—Lyrellales and Cymbellales—in the current classification (Round et al. 1990). This is not wholly surprising. Members of the Lyrellales and Cymbellales have a similar raphe system, with internal central raphe endings that are usually hooked toward the primary side of the valve (although this can be obscured by an overgrowth of silica, e.g. in *Cymbella*: Round et al. 1990, p. 486), volate (flap-like) rather than hymenate pore occlusions (Mann 1981, Round et al. 1990), and constant *cis* symmetry of the frustules (i.e. the primary sides of both valves lie on the same side of the frustule), because the nucleus always divides on the same side of the cell (Mann and Stickle 1988).

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Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. & Donoghue, M. J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Miss. Bot. Garden* 82:247–77.

Beck, A., Friedl, T. & Rambold, G. 1998. Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies. *New Phytol.* 139:709–20.

Bhattacharya, D. & Medlin, L. 1995. The phylogeny of plastids: a review based on comparison of small-subunit ribosomal RNA coding regions. *J. Phycol.* 31:489–98.

Carranza, S., Giribet, G., Ribera, C. & Riutort, M. 1996. Evidence that two types of 18S rDNA coexist in the genome of *Dugesia* (*Schmidtea*) *mediterranea* (Platyhelminthes, Turbellaria, Tricladida). *Mol. Biol. Evol.* 13:824–32.

Chiappino, M. L. & Volcani, B. E. 1977. Studies on the biochemistry and fine structure of silica shell formation in diatoms. VII. Sequential cell wall development in the pennate *Navicula pelliculosa*. *Protoplasma* 93:205–21.

Coleman, A. W. 1979. Sexuality in colonial Volvocales. *In* Hutner, S. H. & Levandowsky, M. [Eds.] *The Protozoa*. Academic Press, New York, pp. 307–40.

Coleman, A. W. 2000. The significance of a coincidence between evolutionary landmarks found in mating affinity and a DNA sequence. *Protist* 151:1–9.

Coleman, A. W. & Mai, J. C. 1997. Ribosomal DNA ITS-1 and ITS-2 sequence comparisons as a tool for predicting genetic relatedness. *J. Mol. Evol.* 45:168–77.

Coleman, A. W., Preparata, R. M., Mehrotra, B. & Mai, J. C. 1998. Derivation of the secondary structure of the ITS-1 transcript in Volvocales and its taxonomic correlations. *Protist* 149: 135–46.

Coleman, A. W., Suarez, A. & Goff, L. J. 1994. Molecular delineation of species and syngens in Volvocacean green algae (Chlorophyta). *J. Phycol.* 30:80–90.

Deduangboripant, J. & Cronk, Q. C. B. 2000. High intraindividual variation in internal transcribed spacer sequences in *Aeschynanthus* (Gesneriaceae): implications for phylogenetics. *Proc. R. Soc. Lond. B* 267:1407–15.

Du Buf, J. M. H. & Bayer, M. M. [Eds.] 2002. *Progress in Automatic Diatom Identification*. Series in Machine Perception and Artificial Intelligence. Vol. 51. World Scientific Publishing Co., Singapore, 316 pp.

Durand, C., Manuel, M., Boudouresque, C. F., Meinesz, A., Verlaque, M. & Le Parco, Y. 2002. Molecular data suggest a hybrid origin for the invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta) in the Mediterranean Sea. *J. Evol. Biol.* 15:122–33.

Elwood, H. J., Olsen, G. J. & Sogin, M. L. 1985. The small-subunit ribosomal DNA gene sequence from the hypotrichous ciliates *Oxytrichia nova* and *Stylonychia pustulata*. *Mol. Biol. Evol.* 2: 399–410.

Famà, P., Olsen, J. L., Stam W, . T. & Procaccini, G. 2000. High levels of intra- and inter-individual polymorphism in the rDNA ITS1 of *Caulerpa racemosa* (Chlorophyta). *Eur. J. Phycol.* 35:349–56.

Friedl, T. 1995. Inferring taxonomic positions and testing genus level assignments in coccoid green lichen algae: a phylogenetic analysis of 18S ribosomal RNA sequences from *Dictyochloropsis reticulata* and from members of the genus *Myrmecia* (Chlorophyta, Trebouxiophyceae cl. nov.). *J. Phycol.* 31:632–9.

Gilmour, J. S. L. & Gregor, J. W. 1939. Demes: a suggested new terminology. *Nature* 144:333.

Glezer, Z. I., Zhuze, A. P., Makarova, I. V., Proshkina-Lavrenko, A. I. & Sheshukova-Poretskaya, V. S. 1974. *Diatomovye vodorosli SSSR, iskopaemye i sovremennye*, vol. 1. Izdatel'stvo "Nauka", Leningrad, 403 pp.

Guillard, R. R. & Lorenzen, C. J. 1972. Yellow-green algae with chlorophyllide c. *J. Phycol.* 8:10–4.

Gutell, R. R., Larsen, N. & Woese, C. R. 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev.* 58:10–26.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95–8.

Hamby, R. K., Sim, L. E., Issel, L. E. & Zimmer, E. A. 1988. Direct RNA sequencing: optimization of extraction and sequencing techniques for work with higher plants. *Pl. Mol. Biol. Rep.* 6:179–97.

Hustedt, F. 1927-66. Die Kieselalgen Deutschlands, Österreichs und der Schweiz unterBerücksichtigung der übrigen Länder Europas sowie der angrenzenden Meeresgebiete. *In Dr L. Rabenhorst's Kryptogamen-Flora von Deutschland, Österreich und der Schweiz*. Vol. 7. Akademische Verlagsgesellschaft, Leipzig.

Ichimura, T. & Kasai, F. 1996. Morphological and cytogenetic characteristics of intergroup hybrids between closely related mating groups of the *Closterium ehrenbergii* species complex (Desmidiales, Chlorophyta). *Phycol. Res.* 44:261–5.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitution through comparative studies of sequence evolution. *J. Mol. Evol.* 16:111–20.

Kooistra, W. H. C. F, Coppejans, E. G. G. & Payri, C. 2002. Molecular systematics, historical ecology, and phylogeography of *Halimeda* (Bryopsidales). *Mol. Phylogen. Evol.* 24:121–38.

Kooistra, W. H. C. F., Stam, W. T., Olsen, J. L. & van den Hoek, C. 1992. Biogeography of *Cladophoropsis membranaceae* (Chlorophyta) based on comparisons of nuclear rDNA ITS sequences. *J. Phycol.* 28:660–8.

Krammer, K. & Lange-Bertalot, H. 1986. Bacillariophyceae. 1. Teil: Naviculaceae. *In* Ettl, H., Gerloff, J., Heynig, H. & Mollen-

hauer, D. [Eds.] *Süßwasserflora von Mitteleuropa*. Vol. 2(1). G. Fischer, Stuttgart and Jena, 876 pp.

Lange-Bertalot, H., Cavacini, P., Tagliaventi, N. & Alfinito, S. 2003. Diatoms of Sardinia. Rare and 76 new species in rock pools and other ephemeral waters. *In* Lange-Bertalot, H. [Ed.] *Iconographia Diatomologica Vol. 12: Biogeography–Ecology–Taxonomy.* A.R.G. Gantner, Ruggell, Liechtenstein, pp. 1–438.

Mai, J. C. & Coleman, A. W. 1997. The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. *J. Mol. Evol.* 44:258–71.

Mann, D. G. 1981. Sieves and flaps: siliceous minutiae in the pores of raphid diatoms. *In* Ross, R. [Ed.] *Proceedings of the 6th Symposium on Recent and Fossil Diatoms*. O. Koeltz, Koenigstein, pp. 279–300.

Mann, D. G. 1984. Observations on copulation in *Navicula pupula* and *Amphora ovalis* in relation to the nature of diatom species. *Ann. Bot.* 54:429–38.

Mann, D. G. 1985. *In vivo* observations of plastid and cell division in raphid diatoms and their relevance to diatom systematics. *Ann. Bot.* 55:95–108.

Mann, D. G. 1989a. The species concept in diatoms: evidence for morphologically distinct, sympatric gamodemes in four epipelic species. *Plant Syst. Evol.* 164:215–37.

Mann, D. G. 1989b. The diatom genus *Sellaphora*: separation from *Navicula*. *Br. Phycol. J.* 24:1–20.

Mann, D. G. 1999. The species concept in diatoms (Phycological Reviews 18). *Phycologia* 38:437–95.

Mann, D. G. 2001. The systematics of the *Sellaphora pupula* complex: typification of *S. pupula*. *In* Jahn, R., Kociolek, J. P., Witkowski, A. & Compère, P. [Eds.] *Lange-Bertalot-Festschrift. Studies on Diatoms, Dedicated to Prof. Dr. Dr. h.c. Horst Lange-Bertalot on the Occasion of His 65th Birthday.* A.R.G. Gantner, Ruggell, pp. 225–41.

Mann, D. G., Chepurnov, V. A. & Droop, S. J. M. 1999. Sexuality, incompatibility, size variation, and preferential polyandry in natural populations and clones of *Sellaphora pupula*. *J. Phycol.* 35:152–70.

Mann, D. G. & Droop, S. J. M. 1996. Biodiversity, biogeography and conservation of diatoms. *Hydrobiologia* 336:19–32.

Mann, D. G. & Stickle, A. J. 1988. Nuclear movements and frustule symmetry in raphid pennate diatoms. *In* Round, F. E. [Ed.] *Proceedings of the 9th International Diatom Symposium*. Biopress, Bristol and O. Koeltz, Koenigstein, pp. 281–9.

Mashkova, T. D., Serenkova, T. I., Mazo, A. M., Avdonina, T. A., Timofeyeva, Y. & Kisselev, L. L. 1981. The primary structure of oocyte and somatic 5S rRNAs from the loach *Misgurnus fossilis*. *Nucleic Acids Res.* 9:2141–51.

Mathews, D. H., Sabina, J., Zuker, M. & Turner, D. H. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* 288:911–40.

Medlin, L. K., Kooistra, W. H. C. F. & Schmid, A.-M. M. 2000. A review of the evolution of the diatoms—a total approach using molecules, morphology and geology. *In* Witkowski, A. & Sieminska, J. [Eds.] *The Origin and Early Evolution of the Diatoms: Fossil, Molecular and Biogeographical Approaches*. W. Szafer Institute of Botany, Polish Academy of Sciences, Cracow, Poland, pp. 13–35.

Mereschkowsky, C. 1902. On *Sellaphora*, a new genus of diatoms. *Ann. Mag. Nat. Hist.*, ser. 7, 9:185–195.

Metzeltin, D. & Lange-Bertalot, H. 1998. Tropische Diatomeen in Südamerika I. 700 überwiegend wenig bekannte oder neue Taxa repräsentativ als Elemente der neotropischen Flora. *In* Lange-Bertalot, H. [Ed.] *Iconographia Diatomologica Vol. 5. Diversity–Taxonomy–Geobotany.*. Koeltz Scientific Books, Königstein, pp. 1–695.

Metzeltin, D. & Lange-Bertalot 2002. Diatoms from the ''Island Continent'' Madagascar. *In* Lange-Bertalot, H. [Ed.] *Iconographia Diatomologica Vol. 11. Taxonomy–Biogeography–Diversity.* A.R.G. Gantner, Ruggell, Liechtenstein, pp. 1–286.

Neefs, J. -M., Van de Peer, V., De Rijk, P., Chapelle, S. & De Wachter, R. 1993. Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res.* 21:3025–49.

Posada, D. & Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14:817–8.

Reimann, B. E. F., Lewin, J. C. & Volcani, B. E. 1966. Studies on the biochemistry and fine structure of silica shell formation in diatoms II. The structure of the cell wall of *Navicula pelliculosa* (Bréb.) Hilse. *J. Phycol.* 2:74–84.

Ritland, C. E., Ritland, K. & Straus, N. A. 1993. Variation in the ribosomal internal transcribed spacers (ITS 1 and ITS 2) among eight taxa of the *Mimulus guttatus* species complex. *Mol. Biol. Evol.* 10:1273–88.

Round, F. E. & Crawford, R. M. 1990. Phylum Bacillariophyta. *In* Margulis, L., Corliss, J. O., Melkonian, M. & Chapman, D. J. [Eds.] *Handbook of Protoctista*. Jones & Bartlett, Boston, pp. 574–596.

Round, F. E., Crawford, R. M. & Mann, D. G. 1990. *The Diatoms. Biology and Morphology of the Genera*. Cambridge University Press, Cambridge, 747 pp.

Rumrich, U., Lange-Bertalot, H. & Rumrich, M. 2000. Diatomeen der Anden von Venezuela bis Patagonien/Feuerland. *In* Lange-Bertalot, H. [Ed.] *Iconographia Diatomologica*. Vol. 9. A.R.G. Gantner, Ruggell, pp. 5–649.

Rzhetsky, A. & Nei, M. 1992. A simple method for estimating and testing minimum-evolution. *Mol. Biol. Evol.* 9:945–67.

Schlösser, U. G. 1994. SAG—Sammlung von Algenkulturen at the University of Göttingen. Catalogue of strains 1994. *Bot. Acta* 107:113–86.

Swofford, D. L. 2001. *PAUP* Phylogenetic Analysis Using Parsimony (*And Other Methods*). Version 4.0b8. Sinauer, Sunderland, MA.

Tamura, K. & Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Bio. Evol.* 10:512–26.

Tang, J., Toè, L., Back, C. & Unnasch, T. R. 1996. Intra-specific heterogeneity of the rDNA internal transcribed spacer in the *Simulium damnosum* (Diptera: Simuliidae) complex. *Mol. Biol. Evol.* 13:244–52.

Taylor, N. G. 1996. *Variation Within* Sellaphora pupula *(Bacillariophyta) in Relation to the Species Concept*. M.Sc. thesis, University of Edinburgh, 52 pp.

VanLandingham, S. L. 1964. Miocene non-marine diatoms from the Yakima region in south-central Washington. *Nova Hedw. Beih.* 14:1–78.

White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* Innes, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. [Eds.] *PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York, pp. 315–22.

Zechman, F. W., Zimmer, E. A. & Theriot, E. C. 1994. Use of ribosomal DNA internal transcribed spacers for phylogenetic studies in diatoms. *J. Phycol.* 30:507–12.

Zuker, M., Mathews, D. H. & Turner, D. H. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. *In* Barciszewski, J. & Clark, B. F. C. [Eds.] *RNA Biochemistry and Biotechnology.* Kluwer Academic Publishers, Dordrecht, pp. 11–43.