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 Sellaphora 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 Sellaphora, as earlier hypothesized for chlorophytes. Intraspecific, 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 phylogeny, 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.
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 Sellaphorinae 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. bacillium* (Ehrenb.) D. G. Mann, and *S. laevisima* (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 sympathy (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. laevisima* 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. laevisima* 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.

<table>
<thead>
<tr>
<th>Taxon and name</th>
<th>Deme</th>
<th>Clone identifier</th>
<th>Collection information</th>
<th>Voucher number</th>
<th>GenBank accession 18S/ITS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sellaphora pupula</em></td>
<td>RECT-1</td>
<td>Rectangular</td>
<td>BS13 Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 19 February 1997</td>
<td>E2997</td>
<td>—/AJ544660</td>
</tr>
<tr>
<td></td>
<td>RECT-2</td>
<td>Rectangular</td>
<td>rect66 Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 12 February 1998</td>
<td>E3161</td>
<td>—/AJ544661</td>
</tr>
<tr>
<td></td>
<td>RECT-4</td>
<td>Rectangular</td>
<td>(BS7)3 Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 19 February 1997; donal F1 progeny reisolated by V. A. Chepurnov after intraclonal auxosporulation</td>
<td>E2996, E3259</td>
<td>AJ544646/AJ544663, AJ544664</td>
</tr>
<tr>
<td></td>
<td>RECT-5</td>
<td>Rectangular</td>
<td>(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</td>
<td>E3004, E3267</td>
<td>—/AJ544665</td>
</tr>
<tr>
<td></td>
<td>RECT-6</td>
<td>Rectangular</td>
<td>(BS14 × BM44)4 Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann and V. A. Chepurnov; 21 September 1997</td>
<td>E3253</td>
<td>—/AJ544666</td>
</tr>
<tr>
<td></td>
<td>PSEUDOCAP-1</td>
<td>Pseudocapitate</td>
<td>18TmNE Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann and V. A. Chepurnov</td>
<td>E3402</td>
<td>AJ544647/AJ544667–AJ544670</td>
</tr>
<tr>
<td></td>
<td>PSEUDOCAP-2</td>
<td>Pseudocapitate</td>
<td>THR42 Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann and V. A. Chepurnov</td>
<td>E3569</td>
<td>AJ544648/AJ544671</td>
</tr>
<tr>
<td></td>
<td>PSEUDOCAP-3</td>
<td>Pseudocapitate</td>
<td>L845.4 Black Dog Lake Creek, Dakota County, Minnesota, USA; coll. D. B. Czarnecki, 19 June 1996 (L845); clonal reisolate by V. A. Chepurnov after intraclonal auxosporulation</td>
<td>E3345</td>
<td>AJ544649/AJ544672</td>
</tr>
<tr>
<td></td>
<td>PSEUDOCAP-4</td>
<td>Pseudocapitate</td>
<td>(L845.5 × THR42)2 Blackford Pond, Edinburgh, Scotland, UK; coll. D. G. Mann, 23 March 1998; clonal F1 progeny reisolated by V. A. Chepurnov after intraclonal auxosporulation</td>
<td>E3384</td>
<td>AJ544650/AJ544673</td>
</tr>
<tr>
<td></td>
<td>CAP</td>
<td>Capitate</td>
<td>BS50 Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann, 19 February 1997</td>
<td>E3220, E3221</td>
<td>AJ544651 (18S + ITS)</td>
</tr>
<tr>
<td></td>
<td>SMALL</td>
<td>Small</td>
<td>8B Blackford Pond, Edinburgh, Scotland, UK; coll. V. A. Chepurnov and D. G. Mann and V. A. Chepurnov; 6 October 1996; nonclonal, following at least one cycle of auxosporulation without reisolation</td>
<td>E3201</td>
<td>AJ544652/AJ544677–AJ544679</td>
</tr>
<tr>
<td></td>
<td>SM-BLCAP</td>
<td>Small blunt-capitate</td>
<td>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</td>
<td>E3161</td>
<td>AJ544653/AJ544674, AJ544675</td>
</tr>
<tr>
<td><em>Sellaphora laevissima</em></td>
<td>SCO</td>
<td>N/A</td>
<td>THR52 Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann, 23 March 1998</td>
<td>E3352</td>
<td>AJ544654/AJ544676</td>
</tr>
<tr>
<td><em>Navicula pelliculosa</em></td>
<td>N/A</td>
<td>A79</td>
<td>N/A West Lake Okoboji, Dickinson County, Iowa, USA; coll. D. B. Czarnecki, 25 August 1982</td>
<td>E3350</td>
<td>AJ544656 (18S + ITS)</td>
</tr>
<tr>
<td><em>Pinnularia cf. interrupta</em></td>
<td>N/A</td>
<td>SAG 1050-3</td>
<td>New Haven Green, Connecticut, USA; coll. J. C. Lewin 1950 Threipmuir Reservoir, near Edinburgh, Scotland, UK; coll. D. G. Mann, 19 January 1997</td>
<td>N/A</td>
<td>AJ544657—/AJ544658—</td>
</tr>
</tbody>
</table>

Voucher material is held in the herbarium at the Royal Botanic Garden Edinburgh (E).
parental clones after intraclonal auxosporation. SM- BLCAP was nonclonal, because it contained the F1 (and F2?) progeny of vigorous intraclonal auxosporation 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 auxosporation 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.) Hils. strain SAG 1050-3 was obtained from the “Sammlung von Algenkulturen” at the University of Göttingen, Germany. A Pennularia 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 Pennularia 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% Na2SiO3, in 50-mL Erlenmeyer flasks. Navicula pelliculosa was grown on diatom agar (Schloesser 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·m–2·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 (Invitек, 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 nt-long motifs, viz. *Mbo* I (MBI Fermentas, St. Leon-Roth, Germany), *Hha* I (Promega, Madison, WI, USA), and *Bol* 11236I (MBI Fermentas).

Cyclic reactions 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 57 °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 (Amer- sham 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 25S- or IRD-labeled and complementary to conserved regions of the 18S, 5.8S, and 26S rDNA coding regions. Plasmids were sequenced using CY5- or IRD-labeled M13 standard primers. Sequencing reactions were run on ALFexpress II (Amerham Pharmacia) and LI-COR L4200 automated sequencers (LI-COR, Lincoln, NE, USA).

Sequences of 18S rDNA for *Pennularia* 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/RNaseF/info/ programs/BIOEDIT/bioedit.html; Hall 1999). 18S rDNA se-quences 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 *Cymatiosira belgica* Grun. (GenBank X85387) and *Papillocellulus elegans* Hasle, von Stosch et Syvertsen (Gen- Bank X85588). 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 rDNA 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphora montana Krasske</td>
<td>AJ243061</td>
</tr>
<tr>
<td>Asterionellopsis glacialis (Castracane) Round</td>
<td>X77701</td>
</tr>
<tr>
<td>Bacillaria paxillifer (O.F. Müll.) Hendley</td>
<td>M87325</td>
</tr>
<tr>
<td>Centrodiscus closterium (Ehrenb.) Reimann et Lewin</td>
<td>M87526</td>
</tr>
<tr>
<td>Eoloma minima (Grun.) Lange-Bertalot</td>
<td>AJ243063</td>
</tr>
<tr>
<td>Eoloma submersula (Manguin) Moser et. al.</td>
<td>AJ243064</td>
</tr>
<tr>
<td>Fragilaria stratiolola Lyngb.</td>
<td>X77702</td>
</tr>
<tr>
<td>Gomphonema parvulum (Kütz.) Kütz.</td>
<td>AJ243062</td>
</tr>
<tr>
<td>Navicula cryptoplephala Kütz.</td>
<td>AJ297724</td>
</tr>
<tr>
<td>Tryblionella apiculata Gregory</td>
<td>M87534</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum Bohlin</td>
<td>AJ269501</td>
</tr>
<tr>
<td>Pseudo-nitzschia multiseries (Hasle) Hasle</td>
<td>U18241</td>
</tr>
<tr>
<td>Pseudo-nitzschia punctata (Grun. ex Cleve) Hasle</td>
<td>U18240</td>
</tr>
<tr>
<td>Rhaphoneis belgica Grun. in Van Heurck</td>
<td>X77703</td>
</tr>
<tr>
<td>Thalassionema nitzschoides (Grun.) Grun. ex Hust.</td>
<td>X77702</td>
</tr>
</tbody>
</table>

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 as equally weighted 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+1+G model (Tamura and Nei 1993) was chosen, with estimates of nucleotide frequencies A = 0.2554, C = 0.1851, 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 = 0.4846); and the proportion of invariant sites that are unable to accept substitutions (pinvar = 0.5088). 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.

<table>
<thead>
<tr>
<th>Clones and intraclonal sequence variants</th>
<th>Length of ITS-1 (bp)</th>
<th>Length of ITS-2 (bp)</th>
<th>Total of positions (incl. indels of 1–3 bp) different among clones / intraclonal sequence variants</th>
<th>Sequence differences within a group of clones or among intraclonal sequence variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>RECT-1, -2, -3, -4a, -4b, -5, -6</td>
<td>358</td>
<td>310–318</td>
<td>25/5</td>
<td>92 positions and 3 indels of 4, 7, and 26 bp</td>
</tr>
<tr>
<td>PSEUDOCAP-1a, -b, -1b, -1c, 1d, -2, -3, -4</td>
<td>261–265</td>
<td>324–354</td>
<td>12/7</td>
<td>18 positions and 2 indels of 35 and 5 bp</td>
</tr>
<tr>
<td>SM-BLCAP-1a, -b</td>
<td>303</td>
<td>313</td>
<td>n.a.</td>
<td>92 positions and 3 indels of 45, 4, and 4 bp</td>
</tr>
<tr>
<td>BLUNT</td>
<td>254</td>
<td>265</td>
<td>n.a.</td>
<td>48 positions and 2 indels of 50 and 4 bp</td>
</tr>
<tr>
<td>CAP</td>
<td>315</td>
<td>286</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>SMALL-a, -b, -c</td>
<td>276/276/229368/367/361</td>
<td>312/355</td>
<td>n.a./102</td>
<td></td>
</tr>
</tbody>
</table>

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.

* RECT-1 differed by a 7-bp insertion in ITS-2 from other RECT clones.
* PSEUDOCAP-1a–d differed by a 9-bp deletion in ITS-2 from other PSEUDOCAP clones.
* 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-1, 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 inter-
mediate between true capitate and rectangular demes 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 PSEUDO-
CAP-2×PSEUDOCAP-3 cross as PSEUDOCAP-4. In 
dimensions, shape, and striation pattern, PSEUDO-
CAP-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 
进一步 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 pseudoca-
pitate demes must therefore be regarded as semicryp-
tic. 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 character-
ized (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.

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

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

*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.

*Measured adjacent to the raphe system, excluding the central area.

*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/adiacdbs.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 striaion (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, 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 epipelagic 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.

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 PSEUDOCAPE-2 cells behaved as males and all PSEUDOCAPE-3 cells as females. Unfortunately, by the time we discovered from molecular data that PSEUDOCAPE-1 (which usually behaved as a male in crosses with RECT clones) was closely related to PSEUDOCAPE-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 PSEUDOCAPE-2 were prevented by the loss of all female RECT clones. The American clone PSEUDOCAPE-3 was capable of intraclonal auxosporulation (as well as outcrossing with PSEUDOCAPE-2), whereas the Scottish PSEUDOCAPE-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 PSEUDOCAPE, there were differences at only two positions and the sequence of just one clone (PSEUDOCAPE-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 PSEUDOCAPE 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 PSEUDOCAPE-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 Thalassiosira and Fragilariopsis 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–Eolimina 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 Sellaphorinae, following Round et al. (1990, pp. 128, 657) (Fig. 3). Anaphora montana and Eolimina subminuscula appeared to be the closest relatives of the Sellaphorinae 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 cryp toxicapha within the raphid pennate diatoms could not be determined. The two genera, Eolimina 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. Anaphora montana (Thalassiosiphales), Lyrella atlantica (Lyellales), and Gomphonema parvulum (Cymbellales). A monophyletic origin of the Bacillariaceae was moderately supported (Fig. 3). When the araphids Thalassiosira and Fragilariopsis 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 PSEUDOCAPE 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-SFO (Table 3).
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

![Maximum likelihood phylogeny of 18S rDNAs from *Sellaphora*, *Navicula pelliculosa*, *Pinnularia cf. interrupta*, *Lyrella atlantica*, and other pennate diatoms.](image)
(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.

**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
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 PSEUDO-DCAP 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 PSEUDO-DCAP 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 rbcL 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. fusiculcus) 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 rbcL 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 intracleade 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 contained 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→U·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×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→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 multiclonal 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) (R biggest 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 (Marshkova 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. laevisima have a single origin, which suggests that Sellaphora, as presently defined, is a monophyletic genus. This conclusion is also supported by rbcL and other ITS 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. cryptoccephala and E. subminuscule are phylogenetically distant from the Sellaphoraceae. The position of N. pelliculosa within the Sellaphoraceae 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 *Pinna- laria* (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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