

# Identification of Heterotrophic Nanoflagellates by Restriction Fragment Length Polymorphism Analysis of Small Subunit Ribosomal DNA

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**ABSTRACT.** Thirty clones derived from twenty isolates of heterotrophic nanoflagellates originating from a variety of marine and freshwater environments were examined by restriction fragment length polymorphism analysis of small subunit ribosomal RNA genes amplified by the polymerase chain reaction (riboprinting). The data were compared with light and electron microscopical identification of the isolates. On morphological criteria, sixteen of the thirty clones belonged to the genus *Paraphysomonas* De Saedeleer, seven to the genus *Spumella* Cienkowski, four to the genus *Pteridomonas* Penard and three to the genus *Cafeteria* Fenchel and Patterson. Among these taxa, eleven ribotypes were detected by analysis with the restriction enzymes *Hinf* I, *Hae* III, *Sau*3A I, and *Msp* I. Differentiation of nanoflagellate taxa by the riboprinting method supported taxonomic classification based on morphology at the generic and species level. The utility of the method for discriminating the ‘naked’ flagellates and for confirming the identity of polymorphic forms among species of *Paraphysomonas* is demonstrated.

**Key Words.** Chrysophyceae, heterokont flagellates, polymerase chain reaction, ribosomal RNA gene, riboprinting.

**H**ETEROTROPHIC nanoflagellates (2–20  $\mu\text{m}$  in diam.) are ubiquitous in aquatic environments where they constitute a major component of the eukaryotic plankton. They are the primary consumers of bacteria and other picoplankton and serve as important nutrient remineralizers in the water column (Azam et al. 1983; Berninger et al. 1991; Caron 1991; Caron et al. 1991; Fenchel 1982). The size of these organisms and their ability to consume bacteria also place the heterotrophic nanoflagellates in a position as trophic links for the transfer of carbon and nutrients from bacteria to higher consumers in planktonic food webs.

Despite their importance in aquatic ecosystems, the diversity and biogeography of many heterotrophic nanoflagellates in natural systems are still poorly known. This is because the size of many of the species is  $\leq 5 \mu\text{m}$ , and the morphological and behavioral characteristics which distinguish individual taxa are not readily apparent. The obscurity of discriminating taxonomic characters is a particular problem among ‘naked’ heterokont flagellates where few morphological features are obvious using light or electron microscopy. This has resulted in a confused and difficult taxonomy for some groups of ‘naked’ heterotrophic nanoflagellates (e.g. within the genera *Oikomonas* and *Spumella*) (Preisig et al. 1991).

Flagellates, which are covered by scales and loricas, present easily discernable criteria for species identification. Scale morphology, for example, is an important character in chrysophyte taxonomy (Kristiansen 1986). Within the chrysophyte genus *Paraphysomonas*, species are distinguished from the ultrastructure of silica scales which cover the cells (Preisig and Hibberd 1982a; Preisig and Hibberd 1982b; Preisig and Hibberd 1983). However, intraspecific variability has been observed in scale size and morphology. The polymorphic scales of *P. imperforata* can show up to 4-fold differences in size (Preisig and Hibberd 1982a; Thomsen 1975; Vørs et al. 1990) while other species may produce imperfect forms. Several species of *Paraphysomonas* normally possess at least two types of scales but these organisms have also been observed with only one scale type. Whether such deviations in scale size and morphology indicate the existence of different species has sometimes been a subject of debate and this has rendered descriptions of some *Paraphysomonas* species rather tenuous (Vørs et al. 1990).

Scale morphology can also be affected by environmental conditions. Silica limitation is known to alter the shape or totally eliminate the production of scales by chrysophytes (Lead-

beater and Barker 1995). The influence of temperature, salinity, and prolonged cultivation on scale morphology has not been well-studied for most taxa. However, these factors may also affect scale production and confound the identification of scaled taxa.

In recent years, rapid identification and classification of microorganisms have been achieved by DNA-based methods. The analysis of small subunit ribosomal RNA (SSU rRNA) gene sequences involving the use of the polymerase chain reaction (PCR), oligonucleotide probes and DNA polymorphism assays, has been especially useful for characterizing microbial populations (Grimont and Grimont 1986; Muyzer et al. 1993; Olsen 1988; Stahl and Amann 1991; Welsh and McClelland 1990; Williams et al. 1990). A method based on the detection of restriction fragment length polymorphisms (RFLP) in SSU rRNA genes amplified by PCR, in particular, has been used successfully to distinguish species and strains of bacteria (Laguette et al. 1994; Laguette et al. 1996; Moyer, 1994), fungi (Molina et al. 1992) and diverse groups of protists such as parasitic and non-parasitic amoebae, myxozoan parasites, *Tetrahymena* ciliates, toxic red tide dinoflagellates of the genus *Alexandrium*, and *Symbiodinium*-like zooxanthellae (Brown and De Jonckheere 1994; Clark 1997; Clark and Diamond 1997; Jerome and Lynn 1996; Rowan and Powers 1991; Scholin and Anderson 1994; Xiao and Desser 2000). This method, also called ‘riboprinting’ (Clark 1997), involves amplification of SSU rRNA gene segments, digestion of the amplified rDNA products with restriction enzymes and separation of the resulting fragments by horizontal gel electrophoresis. This study examines the utility of riboprinting to differentiate clonal cultures of heterotrophic flagellates.

## MATERIALS AND METHODS

**Flagellate cultures.** Thirty clonal cultures (bacterized, but uniprotistan) of heterotrophic flagellates which originated from various freshwater and marine locations along the east coast of the United States, England, and Malaysia were examined (Table 1). All possessed a swimming behavior characteristic of heterokont, chrysomonad-like flagellates. The identity of these species was otherwise unknown at the time that the RFLP analyses were performed, with the exception of *P. imperforata* clone JE1 which was provided by Dr. Jacqueline Eccleston (Lancaster University, UK). Seven of the clones (CRP1, DB1, LBMS1, LW1, PV10, and YP1) were isolates from freshwater ponds and lakes and the remainder originated from marine localities (Table 1). The clonal cultures designated as WH1, WH1a, WH4, and SS were obtained from Dr. John B. Waterbury (Woods Hole

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Table 1. Groupings of heterotrophic nanoflagellates according to small subunit (SSU) rDNA restriction patterns.

Identification (clone name)	Geographic origin	Source <sup>a</sup>	SSU rDNA restriction pattern				SSU rDNA ribotype
			Hinf I	Hae III	Sau3A I	Msp I	
<i>P. imperforata</i>							
WB1-WB5	Waquoit Bay, MA	M	1	1	1	1	I <sup>b</sup>
VS1	Vineyard Sound, MA	M	1	1	2	2	II
JE1	England	M	1	1	2	2	II
SR3	Sakonnet River, RI	M	1	1	2	2	II
<i>Spumella</i> spp.							
EP1, EP2	Eel Pond, MA	M	2	2	2	1	III <sup>c</sup>
YP1	Malaysia	F	2	2	2	3	IV
CRP1	Washington, DC	F	3	3	3	4	V
LW1	Winnepesaukee, NH	F	3	3	3	4	V
LBMS1	Malaysia	F	3	3	3	4	V
MR1	Merrimac River, MA	F	3	3	3	5	VI
<i>P. vestita</i>							
PV10	England	F	4	4	4	6	VII <sup>d</sup>
DB1	Kyle's Pond, MD	F	4	4	4	6	VII <sup>d</sup>
<i>P. bandaiensis</i>							
WH1, WH1a, WH4	Woods Hole, MA	M	5	5	5	7	VIII
SS, Hflag	Sargasso Sea	M	5	5	5	7	VIII
<i>P. butcheri</i>							
DB4	Patuxant River, MD	M	6	6	6	8	IX <sup>b</sup>
<i>Cafeteria</i> sp.							
SR4, SR6	Sakonnet River, RI	M	7	7	7	9	X
Cflag	Woods Hole, MA	M	7	7	7	9	X
<i>Pteridomonas</i> sp.							
NB1, NB2	Narragansett Bay, RI	M	8	8	8	10	XI
SR1, SR5	Sakonnet River, RI	M	8	8	8	10	XI

<sup>a</sup> M = marine isolate, F = freshwater isolate.

<sup>b</sup> An additional PCR product (750 bp) was detected.

<sup>c</sup> An additional PCR product (1000 bp) was detected.

<sup>d</sup> The amplified SSU rDNA was approximately 1950 bp in size.

Oceanographic Institution, MA), PV10 from Dr. Robert W. Sanders (Temple University, PA), DB1 and DB4 were from Dr. Delma Bratvold (George Mason University, VA). The remaining clonal cultures were isolated from enrichment cultures of natural water samples according to the method in Caron (1993) and the procedure briefly described below.

One hundred ml of a water sample was supplemented with sterile yeast extract to a final concentration of 0.01% to promote bacterial growth. These cultures were kept in the dark at room temperature for approximately 7 d to enrich for a mixed assemblage of heterotrophic protists on the bacteria. Subsamples of the enrichment cultures were observed with a Wild M-10 dissecting microscope and individual flagellate cells were picked with a micropipet from a drop of culture. Individual cells were transferred through successive drops of sterile water (to ensure selection of a single cell), and finally transferred to a tube containing a dilute culture of bacteria. After about 2 wk, these clonal attempts were checked for growth of flagellates. Clonal cultures with the same letter prefixes originated from the same enrichment culture and the number following the letters refers to the different clones that were isolated from that enrichment culture.

All the flagellate cultures were maintained at room temperature on either sterilized freshwater or natural seawater supplemented with 0.005% yeast extract and bacteria. Cultures were harvested for microscopic and molecular analyses when the flagellates reached early stationary growth phase (generally, abundances of  $10^4$  to  $10^5$  ml<sup>-1</sup>).

**DNA preparation.** Cells from 1.5 ml of culture were pelleted and resuspended in 50  $\mu$ l of 1  $\times$  PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM MgCl<sub>2</sub>, 0.001% gelatin) supplemented with 0.5% Nonidet P40. The cell suspension was then incubated at 95  $^{\circ}$ C for 10 min to lyse the cells. The supernatant was directly used as template for PCR amplification.

**PCR amplification.** Primers derived from conserved regions flanking the SSU rRNA genes of eukaryotes, designated as Primer A and Primer B, were used for PCR amplification (Medlin et al. 1988). Reactions contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.05% NP40, 200  $\mu$ M of each dNTP, 1 ng  $\mu$ l<sup>-1</sup> of each primer, 0.5  $\mu$ l of Taq DNA polymerase (Promega, Madison, WI) and 3  $\mu$ l of cell lysate (template) in a total volume of 100  $\mu$ l. DNA amplification was performed in a Perkin-Elmer Thermocycler for 30 cycles with the following temperature profile: denaturation at 92  $^{\circ}$ C for 1.5 min, annealing at 55  $^{\circ}$ C for 1.5 min and extension at 72  $^{\circ}$ C for 2 min. Amplified DNA was analyzed by horizontal gel electrophoresis in 1% agarose gels.

**RFLP analysis.** Restriction digests were performed directly on PCR products with a panel of 4-base-recognizing restriction enzymes (*Hae* III, *Hinf* I, *Msp* I, and *Sau* 3A I; New England Biolabs, Beverly, MA). For each organism, PCR product from multiple amplification reactions were pooled for the restriction digests. Aliquots of PCR products (8  $\mu$ l) were digested with 1U of enzyme in 10- $\mu$ l reaction vol. using the manufacturer's recommended buffer and temperature. Digested PCR products were electrophoresed in 3% low melting point agarose (Sigma,

St. Louis, MO) for 2.5 h at 90 V and gels were stained with ethidium bromide (5 ng ml<sup>-1</sup>) for visualization. Fragment sizes were calculated from relative migration distances (compared with DNA size markers; GIBCO BRL, Gaithersburg, MD) using the Bio Image Whole Band Analysis program (Millipore, Bedford, MA). The restriction fragment sizes for each enzyme digest were summed to ensure that they amounted to the approximate size of the undigested PCR product.

**Microscopy.** Live cells were observed with a WILD M10 stereo microscope (Leica) using dark-field optics, and with a Zeiss Standard Compound microscope equipped with phase contrast optics. Cell sizes were not measured from these live observations but from electron micrographs (see below). For transmission electron microscopy (TEM), cultures in late exponential or stationary phases of growth were fixed with glutaraldehyde to a final concentration of 1%. Fixed cells (approximately 1.5 ml) were concentrated to a small vol. by centrifugation, and a drop of concentrate was placed on a formvar-coated copper grid (400-mesh). After allowing the cells to settle for approximately 10 min, excess fluid was removed with a piece of Whatman filter paper and the grid was air-dried. The cells on the grids were then negative-stained with 1% uranyl acetate for 2 min. Grids were subsequently rinsed by quickly immersing them in distilled water, and then air-dried. Microscopy was carried out using a Zeiss 10 transmission electron microscope. The heterotrophic flagellates were identified by flagellar morphology and scale structure (if present), and measurements of their cell size and flagella length were made from electron micrographs. Isolates were identified at the genus and species level where possible.

## RESULTS

**Identification of flagellate clones.** All the flagellates examined in this study are heterotrophic heterokont species, i.e. they lack chloroplasts and possess a forwardly directed hairy flagellum and a second smooth flagellum which sometimes is shorter or exists only as a basal body (Moestrup and Andersen 1991). Twenty-three of the 30 clones were chrysophytes and of these clones, 16 belonged to the genus *Paraphysomonas* De Saedeleer, and seven to the genus *Spumella* Cienkowski. Four clones were pedinellid flagellates belonging to the genus *Pteridomonas* Penard, and the remaining three were bicosoecid flagellates from the genus *Cafeteria* Fenchel and Patterson. A brief description of each group along with species descriptions (where identification was possible) are provided in the following paragraphs.

***Paraphysomonas* De Saedeleer.** *Paraphysomonas* cells possess a long, hairy flagellum and a short, smooth flagellum, characteristic of chrysophytes. Species in this genus are identified by the siliceous scales, which cover the surface of the cell. The group has been studied extensively by Preisig and Hibberd and detailed information on presently defined species of *Paraphysomonas* can be found in these papers (Preisig and Hibberd 1982a; Preisig and Hibberd 1982b; Preisig and Hibberd 1983). Eight isolates were identified as *P. imperforata*, two as *P. vestita*, one as *P. butcheri*, and five as *P. bandaiensis*.

*P. imperforata* Lucas (Fig 1A and B). *P. imperforata* is characterized by scales which consist of a flat baseplate without a thickened rim. A spine arises from the center of the baseplate and ends in an abruptly pointed tip. The clones WB1 to 5 and VS 1 were identified as *P. imperforata* (the strain, JE1, was identified as *P. imperforata* by Dr. J. Eccleston) (Table 1). All were marine isolates. Two originated from different locations in Massachusetts, USA, and one was isolated in England.

The scale type produced by the marine isolate, SR3 (Fig. 1C and D), is different from any described species of *Paraphysomonas*

although the general features of the cell, (cell size  $\approx$  3  $\mu$ m, colorless, heterokont flagellation with one long, hairy flagellum and a short, smooth flagellum) resembles the genus *Paraphysomonas*. The scales are more or less spherical ( $\approx$  1  $\mu$ m) without distinct processes or often with a central accumulation of scale material (Fig. 1D). Some of the scales have an annular fold instead. These scales resemble the aberrant scales sometimes produced by *P. gladiata*, although the aberrant scales of *P. gladiata* have a rim (Preisig and Hibberd 1982a, Fig. 5). The scales of clone SR3 did not possess a rim.

Other evidence from the literature suggests that clone SR3 is a morphological variant of *P. imperforata*. In a study of ultrastructural variability among *Paraphysomonas* species from freshly collected water samples, Thomsen (1975) frequently observed cells that were similar to *P. imperforata* but covered by spineless scales similar to those observed in clone SR3 (Fig. 12 in Thomsen 1975) (Fig. 1C and D). Some of these scales had the annular fold (also present in SR3 scales) noted by Lucas in the original description of *P. imperforata* (Lucas 1967, Plate ID). Thomsen also recorded the presence of groups of "normally" developed *P. imperforata* scales occurring together with spineless scales, indicating that cells covered by spineless scales are likely to be clonal variants of typical *P. imperforata* cells (Thomsen 1975). Based on these descriptions, clone SR3 is at present identified as *P. imperforata* and not *P. gladiata* because of the presence of the annular fold and the lack of a rim on the baseplate.

*P. vestita* (Stokes) de Saedeleer (Fig. 2A and B). The scales of this species consist of a baseplate with a slightly thickened rim. A tapering spine with an acute tip arises from the center of the baseplate. The spines are generally longer relative to the base plate than the spines of *P. imperforata* (Fig. 1B). Two clones, PV10 and DB1, which originated from England and Maryland, USA, respectively, were identified as *P. vestita*. Both were freshwater isolates (Table 1).

*P. bandaiensis* Takahashi (Fig. 2C and D). Scales from marine clones WH1, WH1a, WH4, SS, and Hflag have a baseplate with a thickened rim and a central spine arising from the base plate that ends in a rounded tip. The morphology of the scales resembles that of *P. bandaiensis* Takahashi although Preisig (pers. commun.) has suggested that these clones be considered as small-scaled *P. vestita* on the following basis: i) Small-scaled forms of *P. vestita* may be very similar to *P. bandaiensis* and it is often impossible to distinguish the two species; ii) *P. vestita* was previously reported to have scales (base diam. as small as 0.4  $\mu$ m and a spine length as short as 1.3  $\mu$ m) which overlap with the typical scale dimensions of *P. bandaiensis* (base diameter of 0.2–0.8  $\mu$ m and a spine length of 0.2–1.1  $\mu$ m) (Preisig and Hibberd 1982a); iii) *P. bandaiensis* has previously only been observed in freshwater environments.

The scales of clone WH1, WH1a, WH4, SS, and Hflag have a base diam. that measures  $\approx$  0.6  $\mu$ m and a spine length,  $\approx$  1.0–1.3  $\mu$ m. The dimensions of the scales are intermediate between *P. vestita* and *P. bandaiensis* and therefore cannot be used to determine the identity of these clones. At least one of the clones, WH1, can be grown in freshwater media with bacteria (data not shown) even though the clone was isolated from a marine environment. This finding may invalidate the use of habitat as a criterion for identifying these clones as small-scaled *P. vestita* and not *P. bandaiensis*. It should also be noted that *P. imperforata* and *P. vestita*, which belong to the same species complex as *P. bandaiensis* (species bearing only spine scales with an imperforate base), are commonly found in both marine and freshwater environments. Clones WH1, WH1a, WH4, SS, and Hflag are thus tentatively identified as *P. bandaiensis*.

*P. butcheri* Pennick and Clarke (Fig. 3A and B). This species

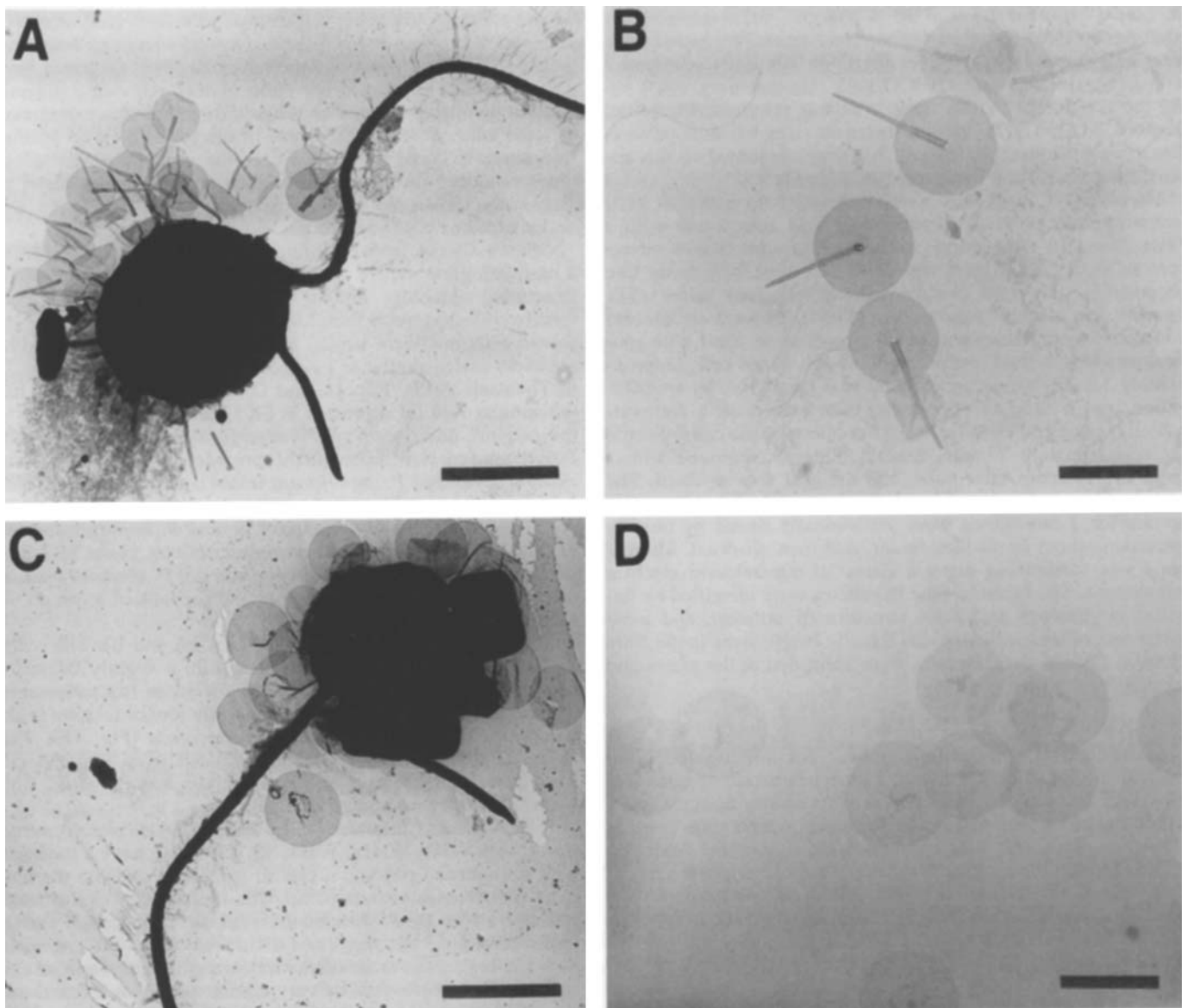


Fig. 1A—D. *Paraphysomonas imperforata*. A: Whole cell, 9,000 $\times$ . B: Group of scales, 17,000 $\times$ . C: Whole cell with spineless scales (Clone SR3), 9,000 $\times$ . D: Group of spineless scales, 13,000 $\times$ . A & C, bar = 2  $\mu$ m; B & D, bar = 1  $\mu$ m.

produces two types of scales. The plate scales are round to elliptical with a meshwork structure, while the crown scales consist of a proximal ring from which a series of five arches arise. These distal arches are connected in the middle by a meshwork structure. The marine isolate DB4 was identified as *P. butcheri* in this study.

*Spumella* Cienkowsky (Fig 3C; D, Fig. 4A, B, C and D). Identification of the naked (i.e. scaleless), chrysomonad flagellates is generally difficult because there are few obvious morphological criteria to distinguish these species. The lack of cellular detail explains the absence of informative taxonomic keys. Most of the descriptions of this genus still date back to original light microscopical observations from the last century (Kent 1880a; Kent 1880b). Identification of the clonal cultures in this study represent the best possible identifications based on current literature.

Isolates identified as *Spumella* are naked, and the long, hairy flagellum and short, smooth flagellum are both visible. Two of

the clones in this study (EP1 and EP2) were coastal marine isolates from Massachusetts while the other five (YP1, CRP1, LW1, LBMS1, and MR1) originated from freshwater localities. These latter five cultures came from ponds and lakes in Washington DC, New Hampshire, Malaysia, and Massachusetts, respectively. Cells of clones EP1, EP2, YP1, and MR1 were  $\approx$  2  $\mu$ m in diam. with a flagellum length of 5–8  $\mu$ m for isolates EP1, EP2, and YP1 (Fig. 3C and D), but the long flagellum of clone MR1 was  $\approx$  10  $\mu$ m (Fig. 4D). The cell diam. of isolates CRP1, LW1, and LBMS1 were all  $\approx$  4  $\mu$ m and the length of their long flagellum was 7–10  $\mu$ m. These latter three isolates have cell bodies that are uniformly covered by an amorphous material that cannot be readily identified from negative-stained preparations (Fig. 4A, B and C). This cell covering gives the outline of the cell body an uneven appearance.

*Cafeteria* Fenchel and Patterson (Fig. 5A and B). A description of this genus and its members based on light microscopical observations of live samples can be found in Larsen and Pat-

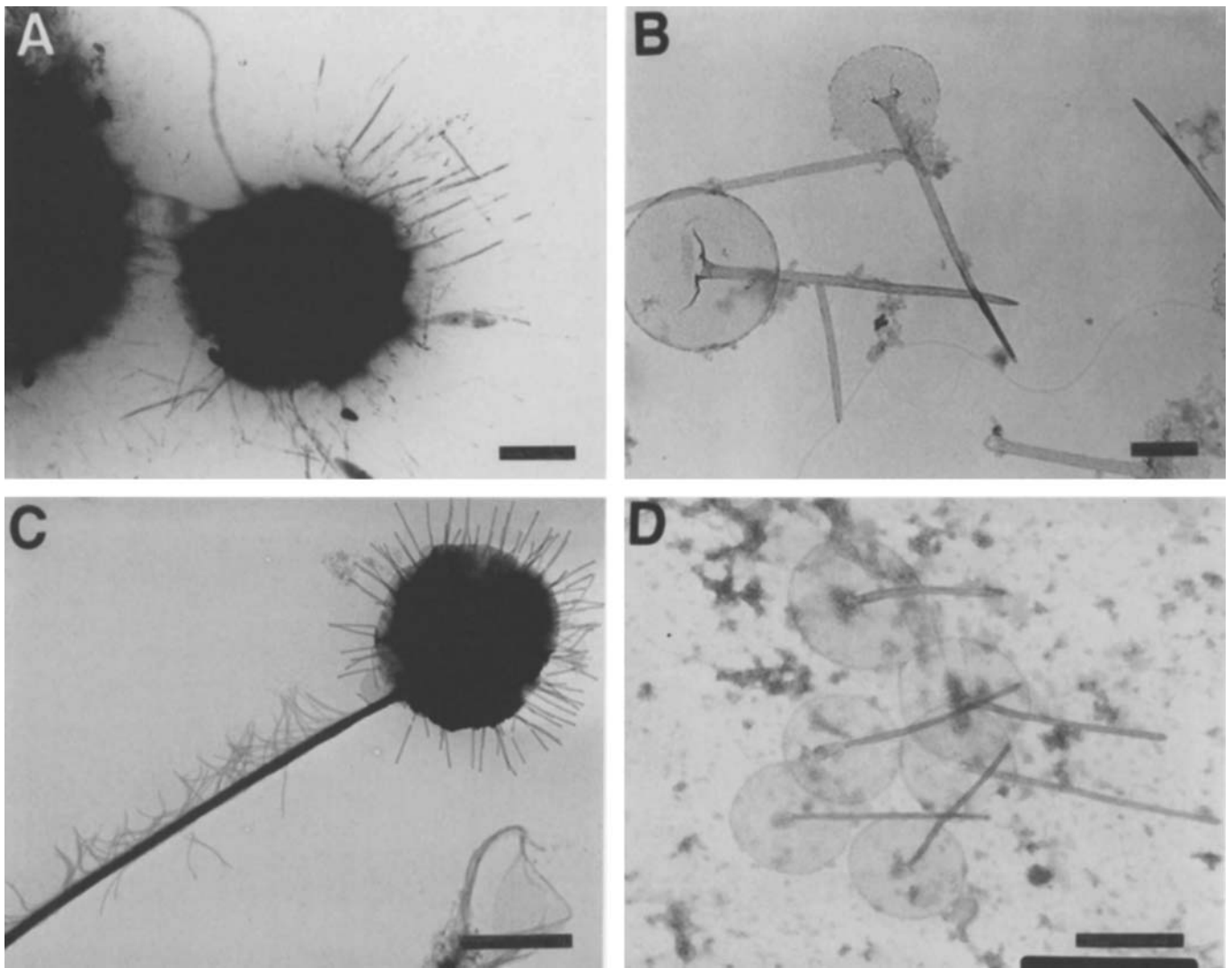


Fig. 2A—D. *Paraphysomonas vestita* and *P. bandaiensis*. **A:** Whole cell of *P. vestita*, 5,500 $\times$ . **B:** Group of *P. vestita* scales, 20,000 $\times$ . **C:** Whole cell of *P. bandaiensis*, 8,000 $\times$ . **D:** Group of *P. bandaiensis* scales, 32,000 $\times$ . A & C, bar = 2  $\mu$ m; B & D, bar = 0.5  $\mu$ m.

terson (Larsen and Patterson 1990). Three clones (SR4, SR6 and Cflag) were identified as belonging to this genus. The generic identity of strain Cflag was confirmed by Dr. D. J. Patterson, based on observations of live samples. In addition, the SSU rRNA genes from clones SR6 and Cflag were sequenced (unpubl.) and were found to be essentially identical to the sequence of *C. roenbergensis* available from GenBank. Strains SR4 and SR6 have spherical to "D"-shaped cell bodies, 2–3  $\mu$ m in diam. The cells possess two flagella of approximately equal length: the hairy flagellum is 6–8  $\mu$ m long and the smooth flagellum is about 7  $\mu$ m long. All three isolates were obtained from marine localities in Rhode Island and Massachusetts.

***Pteridomonas*** Penard (Fig. 5C and D). Cells of clone NB1, NB2, SR1, and SR5 (all originating from marine water samples, Rhode Island) each possess a single hairy flagellum. Cells belonging to the NB clones are  $\approx$  3  $\mu$ m in diam. with flagella that range from 12–15  $\mu$ m in length. The SR clones contain cells 2–3  $\mu$ m in diam. with flagella that are  $\approx$  13  $\mu$ m long. Thin tentacles protruding from the anterior lateral side of the cells are visible by phase contrast microscopical examination but not by TEM. In addition, a stalk on the posterior end of the cell is visible when cells are not motile. This also is not visible

in electron microscopical preparations. On the basis of these observations and those of Patterson and Fenchel (Patterson and Fenchel 1985) and Larsen and Patterson (Larsen and Patterson 1990), clones NB1, NB2, SR1, and SR5 were identified as belonging to the genus *Pteridomonas*, and are most likely the species *P. danica*.

**RFLP analysis of amplified SSU rDNA.** Use of the SSU rDNA primers in PCR yielded amplified rDNA for all the 30 isolates tested. A single band corresponding to an amplification product of approximately 1,800 bp was obtained for all the clones with the exception of WB1 to WB5 and DB4, which yielded an additional  $\approx$  750-bp product; EP1 and EP2, which yielded an additional  $\approx$  1,000-bp product; and PV10 and DB1, which yielded a SSU rDNA product of  $\approx$  1,950-bp instead of the 1,800-bp product. With the exception of DB4, the 1,000-bp and 750-bp products were not visible following enzyme cleavage (Fig. 6). These bands were not included in the riboprinting analysis. The sum of restriction fragment sizes for each enzyme digest was within approximately 10% of the undigested SSU rDNA ( $\approx$  1,650 to 2,000 bp).

Riboprinting of the 30 isolates with *Hinf* I, *Hae* III and *Sau* 3A I resulted in eight distinct cleavage patterns while *Msp* I

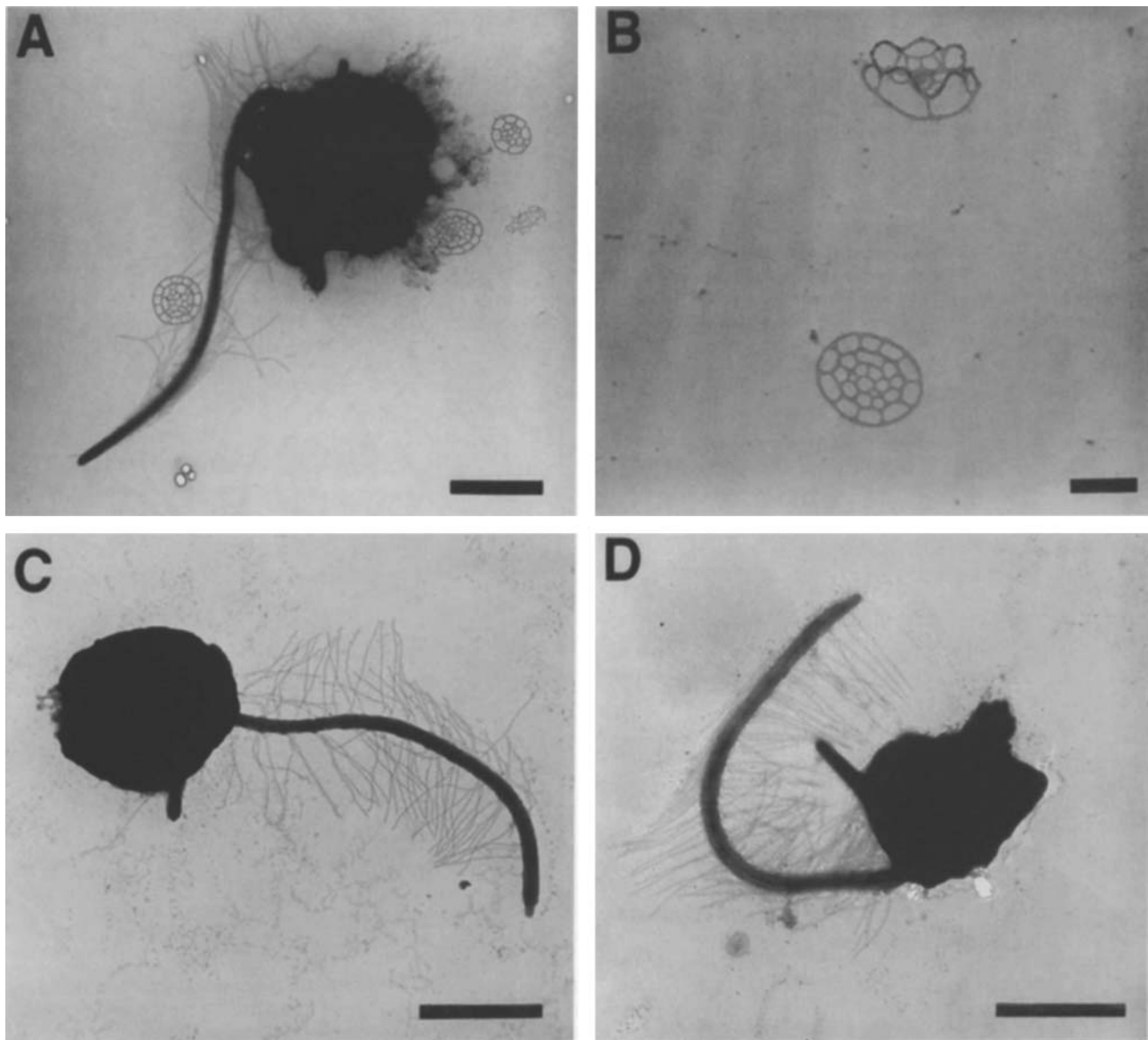


Fig. 3A—D. *Paraphysomonas butcheri* and *Spumella* spp. **A:** Whole cell of *P. butcheri*, 9,000 $\times$ . **B:** Single crown scale (top) and plate scale (bottom) belonging to *P. butcheri*, 20,000 $\times$ . **C:** *Spumella* Clone EP1, 12,000 $\times$ . **D:** *Spumella* Clone YP1, 13,000 $\times$ . A, C & D, bar = 1.5  $\mu\text{m}$ ; B, bar = 0.5  $\mu\text{m}$ .

produced 10 distinct patterns (Table 1). Representative RFLP patterns generated by the enzymes *Hinf* I and *Msp* I are shown in Fig. 6, and are schematically illustrated in Fig. 7 for the enzymes *Hae* III and *Sau* 3A I. The cleavage profiles obtained using the four enzymes allowed us to define SSU rDNA ribotypes, designated I to XI (Table 1). The numbering was assigned arbitrarily and it does not imply any interrelationship among the ribotypes.

The combination of the four enzymes enabled discrimination of the *Paraphysomonas* species, *P. imperforata*, *P. vestita*, *P. bandaiensis*, and *P. butcheri*. Two riboprint patterns were identified among the *P. imperforata* clones: the five *P. imperforata* clones from Waquoit Bay, MA (WB1 to 5), were assigned to one ribotype (I) while three other *P. imperforata* clones, VS1 (Vineyard Sound, MA), JE1 (England), and SR3 (Sakonnet River, RI) with the spineless scales were assigned to a separate ribotype (II). Variations in the SSU rDNA sequences of the *P.*

*imperforata* clones were detected with *Sau* 3A I and *Msp* I but not *Hinf* I and *Hae* III (Table 1). The two ribotypes of *P. imperforata* were distinguished by apparent differences at two *Sau* 3A I sites, and one *Msp* I site (Fig. 6, lanes 1 and 2). The two *P. vestita* clones corresponded to one ribotype (VII). The *P. bandaiensis* clones similarly grouped into a single, unique ribotype (VIII). The riboprint pattern of *P. vestita* differed from that of *P. bandaiensis* for all four enzymes, indicating that the *P. vestita* clones were very distinct from those identified as *P. bandaiensis*. *P. butcheri* was assigned to a ribotype (IX) distinct from the other three *Paraphysomonas* species.

The three genera of naked chrysomonads, *Spumella*, *Cafeteria*, and *Pteridomonas* were separated into ribotypes distinct from each other and from the scaled chrysomonads. Within the genus *Spumella*, the seven clones were differentiated into four ribotypes. Clones EP1 and EP2 were identified as belonging to ribotype III, YP1 as ribotype IV, clones CRP1, LWI, and

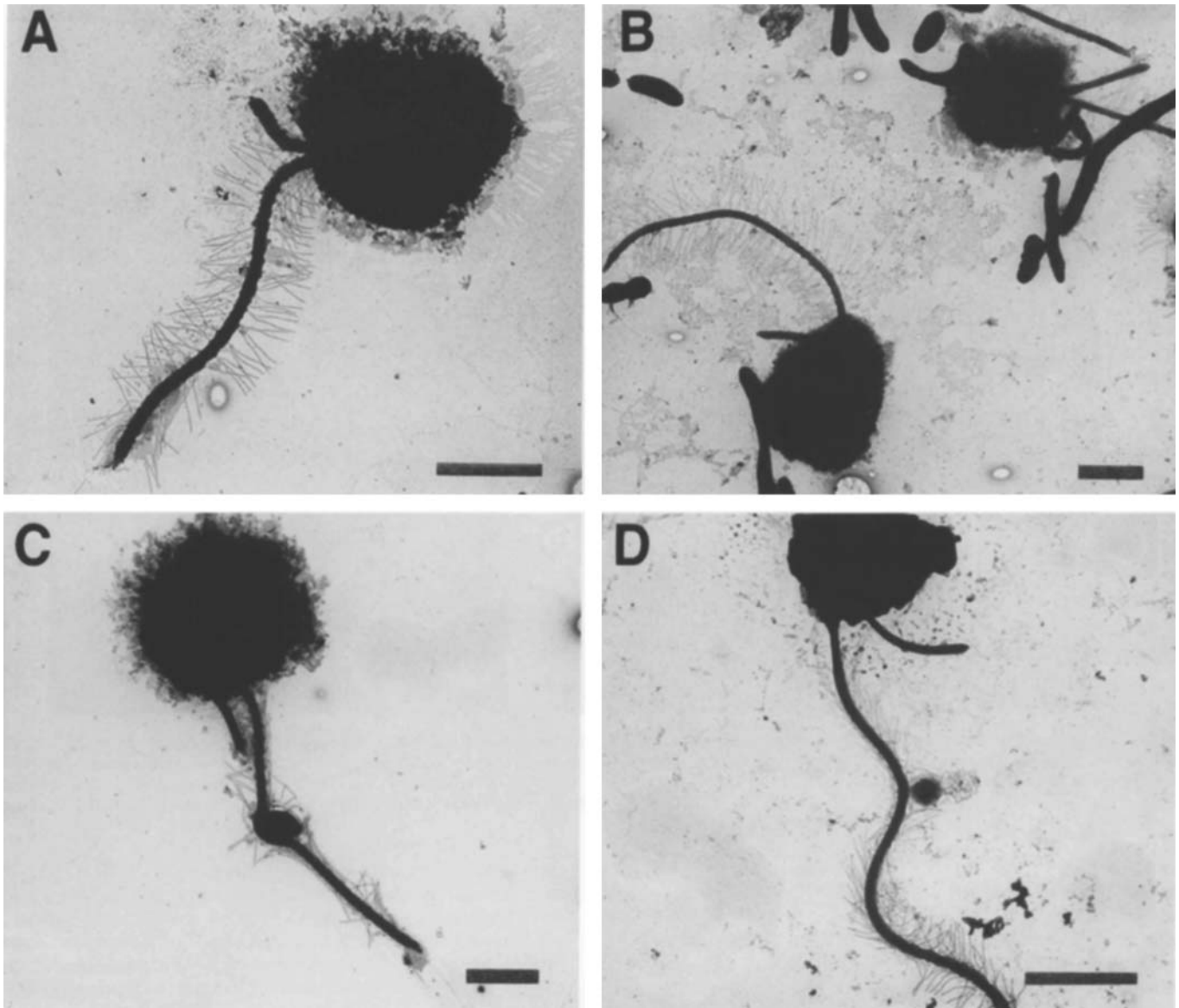


Fig. 4A—D. *Spumella* spp. A: Clone CRP1, 8,000 $\times$ . B: several cells of Clone LW1, 5,000 $\times$ . C: Clone LBMS1, 5,500 $\times$ . D: Clone MR1, 8,500 $\times$ . Bar = 2  $\mu$ m.

LBMS1 as ribotype V, and MR1 as ribotype VI. The three clones ascribed to the genus *Cafeteria* yielded identical riboprint patterns and were assigned to ribotype X. The four clones identified as *Pteridomonas* yielded a single unique restriction pattern assigned to ribotype XI.

A minimum of two enzymes, *Msp* I together with *Hinf* I, *Hae* III or *Sau* 3A I seems capable of providing the same resolving power as the combination of four enzymes. *Msp* I was only slightly less discriminating than the combination of *Msp* I and one of the other three enzymes since 10 of the 11 ribotypes defined from the current panel of clones could be discerned using *Msp* I alone.

#### DISCUSSION

Taxonomic identification of flagellates found in environmental water samples can be complicated even for cultured specimens because of the time and experience needed to distinguish behavioral and morphological features characteristic of the dif-

ferent taxa. This is especially true for the “naked” protists, which lack cell coverings such as body scales or loricas that are often central to protist classification schemes. The utility of RFLP analysis of amplified SSU rDNA, or riboprinting, for examining the phylogenetic relationships of nondescript parasites and amoebae has been widely demonstrated (Brown and De Jonckheere 1994; Clark 1997; Clark and Diamond 1997; Xiao and Desser 2000). In this study, we compared the ability of riboprinting to discriminate heterotrophic flagellates that had been identified by morphological features using conventional light and electron microscopy. The differentiation of isolates by the riboprinting approach showed good agreement with the traditional taxonomic classification based on morphology. Riboprinting provides additional characters that are clearly useful, in combination with morphological features, for discriminating clones of heterotrophic flagellates.

The SSU rRNA gene sequences of the four common genera of heterokont flagellates examined in this study, *Paraphyso-*



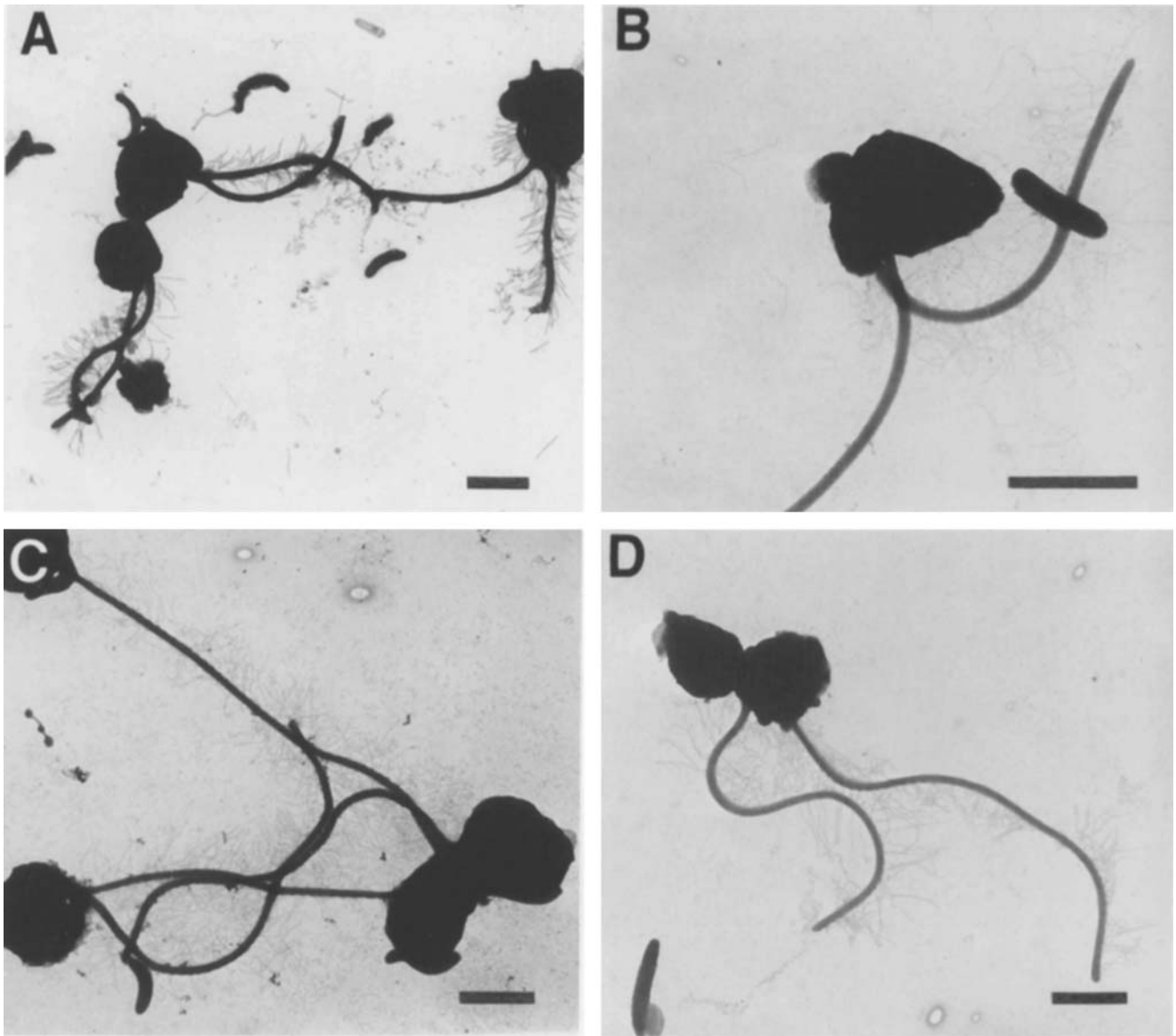


Fig. 5A—D. *Cafeteria* sp. and *Pteridomonas* sp. **A:** several cells of *Cafeteria* Clone SR4, 4,500 $\times$ . **B:** *Cafeteria* Clone SR6, 9,000 $\times$ . **C:** several cells of *Pteridomonas* Clone NB1, 5,500 $\times$ . **D:** two cells of *Pteridomonas* Clone SR1, 5,500 $\times$ . Bar = 2  $\mu$ m.

*monas*, *Spumella*, *Cafeteria*, and *Pteridomonas*, were sufficiently different to permit the discrimination of all four genera. The clear separation of these groups by riboprinting supports their taxonomic distinction based on a combination of flagella and scale morphology. Cells in the genus *Paraphysomonas* and *Spumella* possess a long, hairy flagellum and a second short, smooth flagellum visible in EM preparations. However, *Paraphysomonas* species are surrounded by siliceous scales and *Spumella* species are naked. *Cafeteria* and *Pteridomonas* can be discriminated from *Paraphysomonas* and *Spumella*, and from each other, on the basis of flagellar structure: the smooth flagellum of *Cafeteria* species is typically longer and approximately equal in length with the hairy flagellum, while only a single long, hairy flagellum is visible in TEM preparations of *Pteridomonas*.

Within the genus *Paraphysomonas*, species identification based on scale morphology for *P. imperforata*, *P. vestita*, *P. bandaiensis*, and *P. butcheri* was congruent with riboprinting

separation. This result supports the validity of scale morphology in *Paraphysomonas* taxonomy. More taxa within the genus as well as representatives from other groups of chrysophytes will have to be examined to further verify riboprinting as a reliable complement to scale morphology in the taxonomy of scaled chrysophytes. The ability of riboprinting to discriminate species in the “naked” genera, *Spumella*, *Cafeteria*, and *Pteridomonas*, could not be confirmed because it was not possible to specifically identify the isolates by TEM. However, isolates with similar cell dimensions and flagellar structure within each of these genera were found to cluster into unique ribotypes. These observations provide some evidence to support the hypothesis that riboprinting was able to discriminate species in these genera. The ability of riboprinting to distinguish the “naked”, heterotrophic flagellates is significant because their identification has remained a challenge due to the limited morphological characters on which to base species identification.

Intraspecific sequence heterogeneity was only observed in the



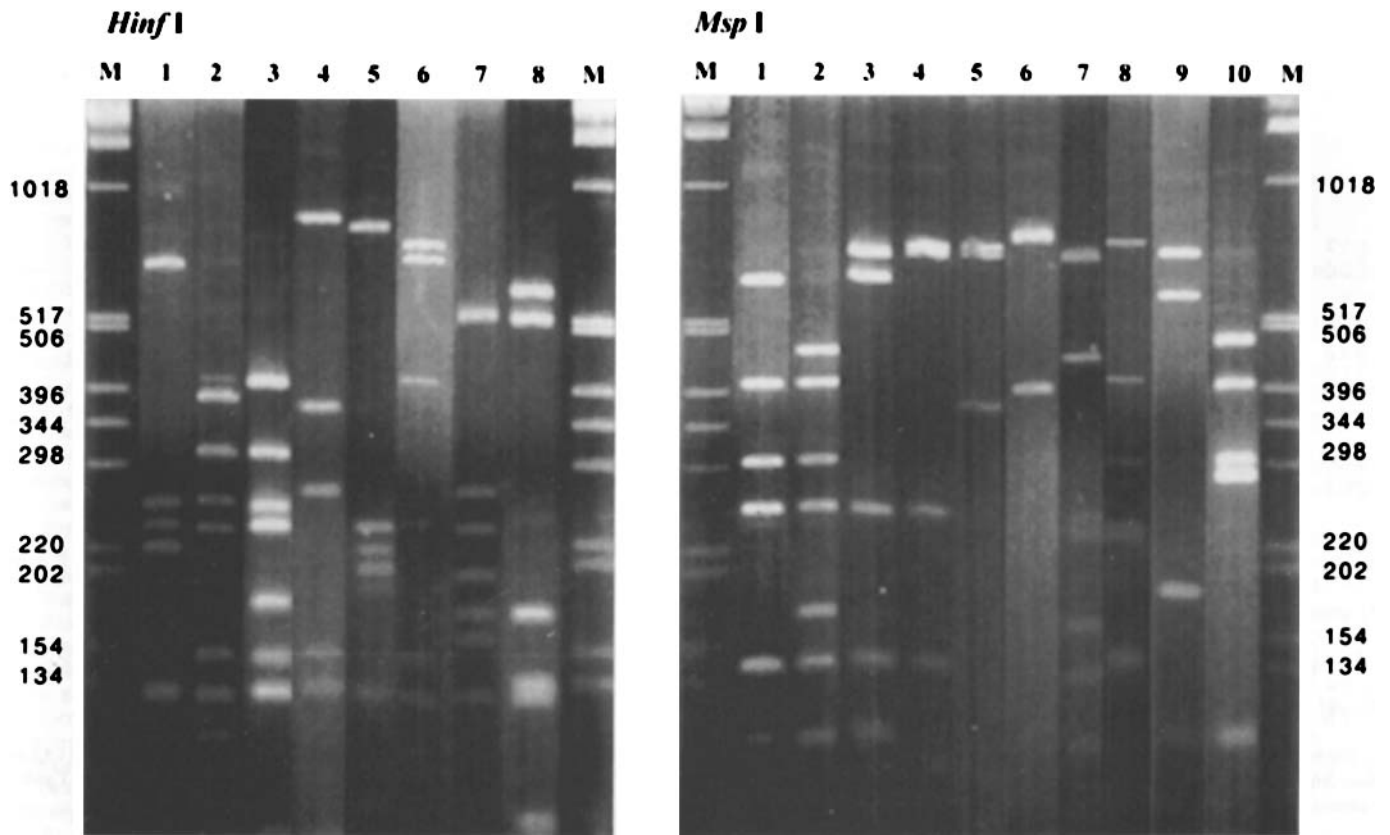


Fig. 6. Restriction patterns of PCR-amplified SSU rDNA observed after incubation with *Hinf* I or *Msp* I. The figure for each enzyme represents a composite image derived from three gels. Migration distances were normalized against the molecular weight markers on each side of the composite gels. The lane assignments (lanes 1–8 for *Hinf* I; 1–10 for *Msp* I) correspond to the restriction patterns for each of the enzymes presented in Table 1. Lane M contained GIBCO BRL "1kb" DNA ladder.

SSU rDNA of *P. imperforata* clones. Clones that were morphologically identical but which exhibited differences in their restriction pattern were defined as possessing intraspecific sequence heterogeneity. Two ribotypes were detected among the seven morphologically identical clones of *P. imperforata*. Full-length sequences of the SSU rDNA of *P. imperforata* clones also indicated the presence of intraspecific sequence variation in this species, as well as in *P. bandaiensis* and *P. vestita* (Caron et al. 1999). However, ribotyping did not detect the sequence variation in *P. bandaiensis* and *P. vestita*. Analysis of more variable regions such as the intergenic spacer region (between the small and large subunit rDNAs) are more appropriate for assessing intraspecific variation and could be analyzed in the future for strain identification.

The ribotyping approach was able to confirm the identity of *Paraphysomonas* clones with atypical scale morphology. Identification of clones WH1, WH1a, WH4, and Hflag as marine specimens of *P. bandaiensis* rather than small-scaled *P. vestita* was supported by RFLP analysis: clones belonging to the *P. vestita* ribotype were distinct from those identified as *P. bandaiensis*, based on the ribotype patterns generated by all four enzymes (Table 1). The discrimination of these two groups could also be correlated to the larger size of the SSU rDNA amplified from *P. vestita* ( $\approx$  1,950 bp) compared to that amplified from *P. bandaiensis* (1,800 bp). The SSU rRNA gene of both *P. vestita* clones has been sequenced in our laboratory and this shows that insertions of "TA-rich" segments (20–100 bp in length) contribute to the larger size of the SSU rDNA in these organisms (data not shown). The distinct SSU rDNA sig-

natures of typical *P. vestita* clones and the marine *P. bandaiensis* clones, based on RFLP analysis and nucleotide sequence data, lend greater confidence to our identification of clones WH1, WH1a, WH4, and Hflag as *P. bandaiensis* and not small-scaled *P. vestita* (Caron et al. 1999). Direct comparison of these clones with a freshwater *P. bandaiensis* type specimen should ultimately resolve this issue.

In the case of *P. imperforata*, the aberrant form with spineless scales (clone SR3) was found to share the same ribotype as *P. imperforata* clones with typical scales. The SSU rRNA genes of clone SR3 and *P. imperforata* clone VS1 have been sequenced in our laboratory (Caron et al. 1999) and the similar sequence data are consistent with the result of the RFLP analysis showing that clone SR3 and clone VS1 are the same species. This finding verifies our identification of clone SR3 [and possibly that of Thomsen's (1975) specimens which had a similar scale type] as *P. imperforata*, and it exemplifies the utility of DNA sequence information for resolving issues of identification raised by scale polymorphism exhibited by *Paraphysomonas* species.

This study has shown that ribotyping is extremely valuable as a parataxonomic tool for discriminating "naked" flagellates from the genus *Spumella*, *Cafeteria*, and *Pteridomonas*, as well as polymorphic forms among species of *Paraphysomonas*. In addition, our analysis demonstrates the importance of using ribotyping in combination with other characters such as scale morphology, cell physiology or complete SSU rDNA sequences to support the interpretation of ribotypes as separate taxa.

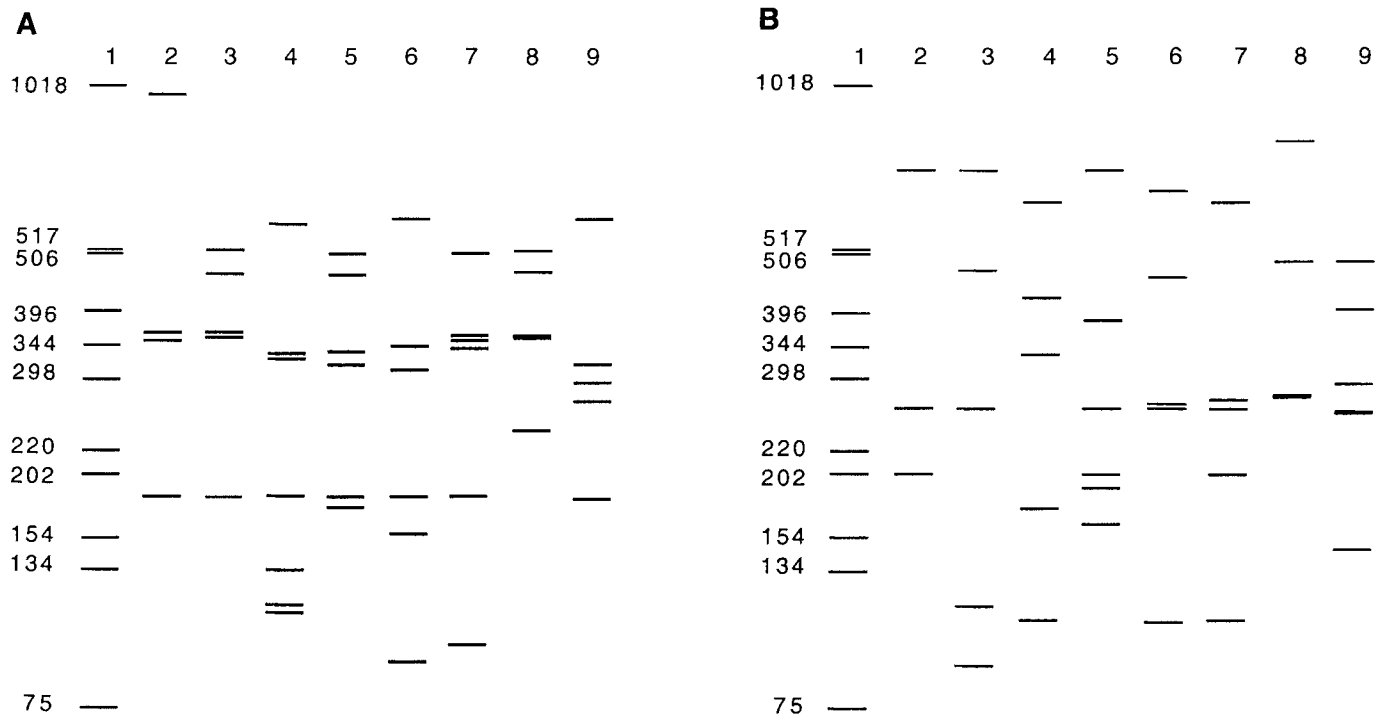


Fig. 7. Schematic representation of restriction patterns obtained from heterotrophic nanoflagellates with the restriction enzyme *Hae* III (A) and *Sau* 3A I (B). Lane 1, DNA size marker; lanes 2–9 correspond to *Hae* III and *Sau* 3A I restriction pattern 2, 1, 7, 6, 3, 5, 4 and 8 in Table 1, respectively.

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