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MOLECULAR PHYLOGENETIC ANALYSIS OF THE HETEROTROPHIC CHRYSOPHYTE GENUS *PARAPHYSOMONAS* (CHRYSOPHYCEAE), AND THE DESIGN OF rRNA-TARGETED OLIGONUCLEOTIDE PROBES FOR TWO SPECIES[1]

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Nanoflagellate protists are algae and protozoa (2–20 μm in size) that play important ecological roles in freshwater and marine microbial communities as primary producers and as consumers of prokaryotic and eukaryotic prey. There is little biogeographical information for most of these minute protists despite their significant role in aquatic food webs. In addition, the evolutionary relationships among some of these species and their affinities to other protistan taxa are unclear. These circumstances are largely a consequence of the fact that small protists possess few readily apparent morphological features on which to base taxonomic and phylogenetic schemes and with which to identify them in natural assemblages. As an alternative approach for addressing these issues, we sequenced the small-subunit ribosomal RNA genes of four species of the colorless chrysophyte genus *Paraphysomonas*. A phylogenetic analysis based on that sequence information was performed, and oligonucleotide probes for two commonly occurring species of *Paraphysomonas* were designed and tested. Phylogenetic analyses of these four species confirmed the affinity of the genus *Paraphysomonas* with other chrysophyte species. High sequence similarity among three of the species (*P. imperforata* Lucas, *P. bandaiensis* Takahashi, and *P. foraminifera* Lucas) supported a previous phylogenetic grouping of these species based on the morphology of the scales produced by these species. In particular, sequence similarity between *P. imperforata* and *P. foraminifera* indicated that this speciation was a recent evolutionary event. However, a fourth species (*P. vestita* (Stokes) de Saedeleer) possessing similar scale morphology to *P. bandaiensis*, *P. imperforata*, and *P. foraminifera* showed considerable sequence dissimilarity in comparison to these latter three species. Oligonucleotide probes were successfully designed for the species *P. imperforata* and *P. bandaiensis* and applied together with a recently developed quantitative *in situ* hybridization procedure. The development of species-specific oligonucleotide probes for these nanoflagellate species and their application for counting nanoflagellates in natural water samples provide tools for studying these ecologically important species.

Key index words: **18S rRNA; chrysophyte; *in situ* hybridization; microbial ecology; nanoflagellate; oligonucleotide probe; *Paraphysomonas*; protistan biogeography; protistan phylogeny; protozoa**

Phototrophic and heterotrophic nanoplankton (usually abbreviated PNAN and HNAN, respectively) are important microbial assemblages in marine plankton communities. Phototrophic nanoplankton from a wide variety of microalgal taxa occur in aquatic ecosystems and are often a dominant portion of the biomass and primary production of marine and freshwater communities (Malone 1971, Booth et al. 1982, Estep et al. 1984, Davis et al. 1985, Joint et al. 1986, Pick and Caron 1987, Wehr 1990). Nanoplanktonic protozoa are important in the cycling of matter and energy in aquatic ecosystems as consumers of prokaryotes and minute eukaryotes, as nutrient remineralizers, and as links to higher trophic levels (Azam et al. 1983, Sherr et al. 1986, Stoecker and Capuzzo 1990, Caron and Finlay 1994, Sherr and Sherr 1994).

Modern representations of microbial food webs typically group assemblages of microorganisms into ecological ''compartments'' in order to reduce the immense biodiversity of microbial communities to a manageable level of complexity. These compartments are defined most generally by cell size and the presence or absence of chloroplasts based on the assumptions that most predator–prey relationships are size dependent and that phototrophy and heterotrophy represent fundamentally (and exclusively) distinct trophic modes (Sieburth et al. 1978, Azam et al. 1983, Sherr and Sherr 1988, Ducklow 1994, Caron 1997). Accordingly, most species of

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planktonic protists are divided into groups based on cell size (e.g. 2–20 μm = nanoplankton; 20–200 μm = microplankton) and phototrophic capacity (e.g. PNAN, HNAN).

Whereas this division of species into trophic compartments or ''guilds'' can be a useful approach for first-order analyses of ecosystem function (Ducklow 1994), there is clear evidence that in many cases these distinctions are artificially contrived. For example, some protistan predators are capable of consuming prey that are larger than their own cell dimensions (Jacobson and Anderson 1986). In addition, numerous species of phototrophic nanoplankton are capable of simultaneously conducting phagotrophy and photosynthesis, and many planktonic (heterotrophic) ciliates possess the ability to ingest and digest algal prey while retaining the chloroplasts of these prey in a functional state (Jones 1994, Stoecker 1998). Occurrences of these types of mixed nutrition among protists appear to be the generality in many microbial food webs rather than the exception. The existence of these trophies that transcend current descriptions of microbial food webs necessitates new characterizations of food web structure that are based on the actual trophic mode(s) of the constituent species.

Most currently used methodologies for enumerating PNAN and HNAN in natural samples are based on epifluorescence or electron microscopical techniques. The former involve the use of fluorochrome stains to visualize protistan cells and the autofluorescence of chlorophyll *a* to distinguish pigmented (supposedly nonphagotrophic) protists from nonpigmented ones (Sherr et al. 1993). These methods are, in large part, responsible for our present awareness of the importance of small protists in aquatic food webs. Species (or even generic) identifications, however, typically are not possible using epifluorescence microscopy because many of the taxonomic features of these organisms are visible only by electron microscopy or in live specimens (e.g. swimming behavior).

Electron microscopy also has been employed as an effective means of identifying many small protists in natural water samples (Leadbeater 1993). This approach allows resolution of fine structural details of small protists upon which modern taxonomies and systematics rely (e.g. mitochondrial structure, body scales). Unfortunately, electron microscopy is relatively costly and time consuming and, thus, not feasible for large numbers of samples that often are collected within the context of ecological or biogeographical studies. More importantly, considerable sample concentration, enrichment, and preparation usually are necessary to obtain sufficient material for analyzing natural water samples by electron microscopy. Therefore, whereas electron microscopy provides some complementary taxonomic data to epifluorescence microscopy, it generally doesn't provide quantitative information on the abundances of small protists. The combined use of these methods is powerful but rarely conducted because of the labor-intensive nature of these endeavors (Estep et al. 1984).

An alternative approach to morphologically based schemes for species identification of small protists is the use of DNA sequence information. In particular, the use of 16S-like (18S) small-subunit ribosomal RNA genes (SSU rRNA) recently has become popular for addressing issues of evolution and taxonomy among these single-celled organisms. Sequence data have been used as clinical and ecological tools to distinguish between morphologically similar species, to trace the movement of environmentally damaging or pathogenic species, or as an alternative means of identifying species with few descriptive morphological features (McLaughlin et al. 1992, Fell 1993, Gast and Caron 1996, Knauber et al. 1996, Simon et al. 1997). Sequence data also have been employed recently in conjunction or in comparison with classical morphological criteria for characterizing phylogenetic relationships among protistan taxa (Bhattacharya et al. 1992, Andersen et al. 1993, Hinkle and Sogin 1993, Leipe et al. 1994, Patterson 1994).

Our goal in this study was to develop new tools for conducting studies of the ecology of heterotrophic nanoplanktonic protists within the colorless chrysophyte genus *Paraphysomonas.* Species within this genus are common in marine and freshwater plankton communities where they are believed to play an important role as consumers of small algae, bacteria, and cyanobacteria (Thomsen 1975, Takahashi 1976, Fenchel 1982c, Preisig and Hibberd 1983, Andersen and Sørensen 1986, Hällfors and Hällfors 1988, Nicholls 1989, Turley and Carstens 1991, Wujek and O'Kelly 1992, Vørs 1993, Hansen 1996). *Paraphysomonas* species are commonly observed in concentrated or enriched natural water samples. Moreover, species from this genus have been employed repeatedly in physiological studies of small protozoa (Fenchel 1982b, Davis and Sieburth 1984, Caron et al. 1985, 1986, 1990, Goldman and Caron 1985, Goldman et al. 1985, Andersen et al. 1986, Landry et al. 1991, Turley and Carstens 1991, Peters et al. 1996). Studies of these species therefore may have considerable ecological relevance.

We have previously reported on the application of SSU rRNA-targeted probes for counting small protists in natural samples (Lim et al. 1993) and on the development of a quantitative *in situ* hybridization method for applying these probes (Lim et al. 1996). In this manuscript, we provide SSU rDNA sequence information for four species of *Paraphysomonas,* a molecular phylogeny of this genus based on our sequence data, and our results on the design and testing of probes toward two of these species (*P. imperforata* Lucas and *P. bandaiensis* Takahashi). A companion manuscript reports the first quantitative data obtained using these probes on the natural

TABLE 1. Clonal designations and origins of *Paraphysomonas* species sequenced in this study.

Species	Clone designation	Origin	Source
Paraphysomonas bandaiensis	WH1	Woods Hole, MA	J. W. Waterbury
Paraphysomonas bandaiensis	Hflag	Sargasso Sea	R. J. Olson
Paraphysomonas imperforata	VS1	Vineyard Sound, MA	D. A. Caron
Paraphysomonas imperforata	VS2	Vineyard Sound, MA	D. A. Caron
Paraphysomonas imperforata	SR3	Sakonnet River, RI	D. A. Caron
Paraphysomonas vestita	PV10	CCAP, UK[a]	R. W. Sanders
Paraphysomonas butcheri	DB4	Patuxant River, MD	D. Bratvold

[a] Clone PV10 was reisolated from a contaminated culture originally obtained from the Culture Collection of Algae and Protozoa (CCAP), Cumbria, United Kingdom as clone 935/14.

abundance of *P. imperforata* in coastal waters of the North Atlantic (Lim et al. 1999).

MATERIALS AND METHODS

Cultures and species identification. The cultures of *Paraphysomonas* species used in this study were established from natural freshwater and seawater samples using enrichment and single-cell isolation techniques (Caron 1993), from other investigators, or from commercial culture collections (Table 1). Uniprotozoan (i.e. bacteria present) clonal cultures were established from enriched natural water samples obtained by collecting samples in sterile containers, enriching with yeast extract broth (final enrichment 0.001–0.005%) or with sterile rice grains to promote bacterial growth in the samples, and incubating in the dark at 20° C. The enrichments were examined periodically for growth of heterotrophic flagellates. Protistan cultures were established from these enrichments by micropipetting individual cells through three washes of sterilized seawater, then placing them into medium with bacteria. Cultures acquired from other investigators or culture collections were subjected to the micropipetting method or serial dilution/extinction to ensure that they were clonal.

Species identifications for all of the *Paraphysomonas* strains used in this study (including strains from private and commercial culture collections) were verified by transmission electron microscopy and published descriptions (Preisig and Hibberd 1982a, b, 1983). Identifications of *Paraphysomonas* species were based on the morphology of the siliceous body scales that characterize these species (see Results; Fig. 1). The complete SSU rRNA genes of four species of *Paraphysomonas* were sequenced in this study and used for probe design and testing: *P. imperforata, P. bandaiensis, P. butcheri* Pennick et Clarke, and *P. vestita* (Stokes) de Saedeleer. Multiple strains of each species were sequenced where possible in order to document intraspecific sequence variability. Three strains of *P. imperforata* (isolated from two locales), two strains of *P. bandaiensis* (from two locales), one strain of *P. vestita,* and one of *P. butcheri* were sequenced.

Nucleic acid extraction, purification, sequencing, and sequence alignment. Cultures of *Paraphysomonas* were grown to the late exponential growth phase (cell densities generally $\geq 10^5$ cells·mL^{-1}) on a mixed bacterial flora. Protistan cells were pelleted by centrifugation and washed, and total nucleic acids were extracted and purified by standard methods (Sambrook et al. 1984). Small-subunit rDNA for sequencing was obtained from this product by polymerase chain reaction (Saiki 1988) using primers that target conserved flanking regions of the gene (Medlin et al. 1988). Two amplifications were performed for each product, with the use of either a forward or reverse biotinylated primer in combination with an unlabeled primer. The biotinylated strand was then isolated with the use of streptavidin-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway) and sequenced directly by manual techniques (Sequenase; USB, Cleveland, Ohio) with the use of standard SSU rDNA sequencing primers (Elwood et al. 1985, Sogin and Gunderson 1987, Medlin et al. 1988). Complete sequencing of the forward and reverse strands was performed, and sequences were assembled using BioImage DNA Sequence Film Reader software (Millipore). Alignment of the sequences obtained in this study with each other and with sequences from GenBank was performed by eye using Genetic Data Environment software (version 2.2; Steven Smith, University of Illinois).

Phylogenetic analyses. Phylogenetic analyses were performed on seven strains (within four species) of *Paraphysomonas* sequenced in this study (GenBank accession numbers AF109322–AF109326), together with SSU rDNA sequence information of another *P. vestita* strain and of *P. foraminifera,* available through GenBank (accession numbers Z28335 and Z38025, respectively). Two different data sets were used. The first included sequences for five *Paraphysomonas* species (one strain from each species) with an expanded set of outgroup sequences in order to obtain a ''global'' perspective of the genus. The second data set used all of the *Paraphysomonas* sequences available but limited the outgroups to the Synurophyceae and the Chrysophyceae to define the branch order within the genus. Positions of dubious homology were eliminated from both of the alignments, and this included most gaps. Any gaps that remained were treated as missing data. The SSU rDNA sequence for *P. vestita* had large A/T-rich insertion sequences that were not included in either of the data sets. All analyses were run using PAUP beta version 4.0.0d64 (Sinauer Associates, Sunderland, Massachusetts) and all trees were unrooted but constructed with reference to an outgroup sequence.

Phylogenetic reconstructions were generated by maximum likelihood (M-L). Due to the large number of taxa in the global data set, an initial M-L analysis was run where the starting trees were obtained by neighbor joining followed by tree bisection and reconnection branch swapping. Two best trees were obtained out of 30,049 possible rearrangements. From those first trees, a backbone was constructed using the branch order of the taxa outside of the chrysophyte/synurophyte lineage. This backbone was used as a constraint for M-L runs using 10 random sequence addition replicates. Out of the 83,496 rearrangements, the three best trees were obtained. Two of these trees were the same as the initial trees.

Maximum likelihood analyses of the data set for the *Paraphysomonas* genus were run with starting trees generated by stepwise addition with the random addition of sequences in 10 replicates, followed by branch swapping with tree bisection and reconnection. Only one best tree was obtained in each of the replicates (out of 68,952 total rearrangements).

Parsimony was used to generate bootstrap values for both data sets with 1000 resamplings. Starting trees were obtained by stepwise addition of taxa with a random addition of sequences (10 times for each bootstrap resampling) followed by tree bisection and reconnection branch swapping. Bootstrap values from the parsimony analyses are shown at their corresponding nodes on the trees.

Probe design, testing, and application. The choice of the SSU rDNA for the oligonucleotide probe design is favored by a number of features of these genes, including the existence of interspersed conserved and variable regions (to facilitate alignment and provide regions for probe design, respectively), high target number in cells, and a reasonably large sequence database for sequence comparison and probe design.

Oligonucleotide probes, complementary to regions of the SSU rRNA, were designed by visual examination of the aligned sequences of the *Paraphysomonas* species and a variety of phyloge-

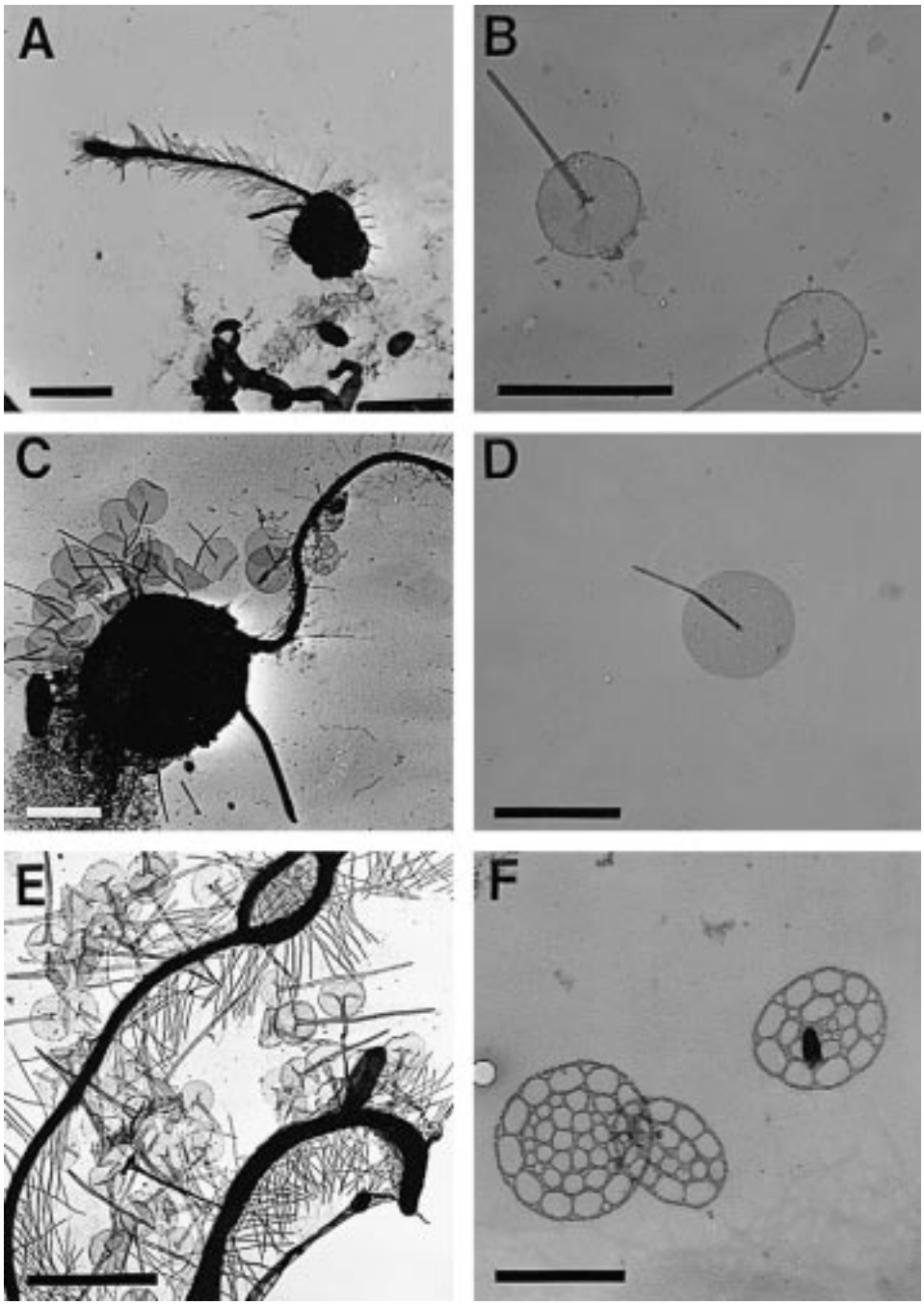

FIG. 1. Electron micrographs of diagnostic features of four *Paraphysomonas* species. Whole-cell and scale morphologies of the species *P. bandaiensis* (A, B) and *P. imperforata* (C, D), and scale morphologies for *P. vestita* (E) and *P. butcheri* (F). Solid plate-like spines produced by *P. bandaiensis* (B), *P. imperforata* (D), and *P. vestita* (E) differ in features of the spine (length, termination) and in a thickened rim at the edge of the plate (present in *P. bandaiensis* and *P. vestita* but absent in *P. imperforata*). *Paraphysomonas butcheri* produces plate-like and crown-like scales composed of a meshwork. Markers bars = 3.0 μm (A, C, E) or 1.0 μm (B, D, F).

netically related species in the sequence database (Table 2). Probe designs, 18–22 base pairs in length, were tested using CHECK PROBE in the RDP and BLAST sequence similarity searching in GenBank. The resulting oligonucleotide probes were synthesized with an amino group at the 5′ terminus (Operon Technologies, Inc.). Probes were labeled at this site either with ^{32}P (^{32}P-ATP and polynucleotide kinase) for examination of dissociation temperatures and probe specificity or with biotin (Molecular Probes, Inc.) for use in whole-cell hybridizations. Biotin-labeled probes were purified of excess biotin using NENSORB 20 columns (Dupont®), then band isolated on 20% polyacrylamide Long Ranger® gels (AT Biochem, Malvern, PA).

Dissociation temperatures were determined empirically by testing each probe against nucleic acids extracted from the target species and closely related species. Nucleic acids were blotted and then UV-cross-linked to nylon membranes. Each blot included nucleic acids from two clones of *P. bandaiensis* and *P. imperforata,* and one clone of *P. vestita, P. butcheri, Spumella* sp., and *Dinobryon cylindricum.* Labeled probes (^{32}P) were tested at eight wash temperatures ranging from 30°–65° C.

The specificities of the oligonucleotide probes were subsequently tested against a variety of extracts of total nucleic acids from target and nontarget organisms (chrysophyte, eustigmatophyte, synurophyte, diatom, chlorophyte, prasinophyte, euglenophyte, prymnesiophyte, rhodophyte, dictyophyte [pedinellid], bicosoecid, kinetoplastid [bodonid], ciliate, and a eubacterium). Nucleic acids from this diverse array of nanoplanktonic algae and protozoa (and one bacterial strain) were extracted, and approximately 50-ng aliquots of DNA of each species was blotted onto nylon filters, dried, and UV-linked. Replicates of these ''zooblots''

TABLE 2. Oligonucleotide probes designed for in situ hybridization with *Paraphysomonas bandaiensis* (Pband), *P. imperforata* (Pimp), and the *P. bandaiensis, P. imperforata, P. vestita* species group (Pbiv). Target region identifications refer to sites in *Ochromonas danica.* Dissociation temperatures (Td) are °C with 150 mM Na^+.

Probe	Sequence (5′–3′)	Target region	Td
Pband 635	TGA GGG ATG GAC CGG TTG CC	635–653	53.4
Pimp 635	TGA GGG GCG GAC CGG TCG CC	635–653	59.6
Pband 663	GGC CGC AGA AAC CTG GTA CAC A	658–679	56.6
Pimp 663	GGA CGC AGA GAC CAG GTG CAC A	658–679	56.6
Pband 1683	CCG ATC CGC GGT CCG AAA	1666–1683	49.6
Pimp 1683	CCA AGC CGC AGT CCG AGA	1666–1683	49.6
Pband 706	CCC ACA CCA GAC AAC TCA AT	704–723	47.3
Pbiv 1537	CAA GAT TCA GAA TTG CAA AAA	1539–1559	40.5

were probed with eukaryote-specific probe Euk 1209 (Sogin and Gunderson 1987), to provide a means of assessing the relative amounts of DNA bound to the filters, then with each of the probes. Hybridizations were performed with ^{32}P-labeled probes. The zooblots were hybridized for at least 4 h (or overnight) at appropriate temperatures then washed with 1× SET solution for 0.5 hour at room temperature then for 0.5 hour at a stringent temperature (see Fig. 4). Hybridization and wash temperatures were based on the dissociation temperatures obtained previously.

Whole-cell hybridizations with oligonucleotides. Probes were employed for *in situ* hybridizations with cultures of *P. bandaiensis* and *P. imperforata,* and with mixed cultures of these species to confirm probe specificity in whole cells and to examine signal strength of probed protists. We have previously established that a single biotinylated probe provides sufficient signal for cultured or enriched protozoa in the exponential or early stationary growth phases (Lim et al. 1993). However, multiple biotinylated probes (two or three) used simultaneously ensured visualization of all target cells in environmental samples (Lim et al. 1993). Therefore, we probed cultures simultaneously with three oligonucleotides possessing similar dissociation temperatures. Probes Pband 635, Pband 663, and Pband 1683, which targeted *P. bandaiensis,* were used simultaneously, as were probes Pimp 635, Pimp 663, and Pimp 1683, which targeted *P. imperforata.* Three eukaryote-specific probes, Euk 309, Euk 502, and Euk 1209 (Sogin and Gunderson 1987), were employed simultaneously as a positive control. A hybridization temperature of 40° C and a wash temperature of 45° C were used for these applications. In practice, hybridization and wash temperatures below the dissociation temperature provide a strong signal from the target with little nonspecific hybridization (DeLong 1991). Specificity for the probes at these hybridization and wash temperatures was investigated empirically with whole-cell hybridizations of single and mixed cultures of *Paraphysomonas* to confirm that the probe combinations effectively probed target species but did not cross react with closely related species.

Protists were preserved with formaldehyde at a final concentration of 3.7% and stored at 4° C in the dark until probing. Biotin-labeled probes were applied according to a recently described quantitative *in situ* hybridization protocol (Lim et al. 1996). Briefly, the preserved sample is drawn down onto a polycarbonate filter (0.4 μm pore size) attached to the bottom of a Transwell® cell culture insert (Costar, Cambridge, Massachusetts), which allows retention of the cells but exchange of solution. Samples were dehydrated through an ethanol series, prehybridized in hybridization buffer (10× Denhardt's solution, 0.1 $mg \cdot mL^{-1}$ polyadenylic acid, 5× SET buffer, 0.1% sodium dodecyl sulfate) for 45 min at 40° C then probed overnight at 40° C. Each probe was used at a final concentration of 2.5 $ng \cdot \mu L^{-1}$. Samples were washed in 0.2× SET for 10 min at 45° C following hybridizations, and bound probe was detected using buffered avidin (20 $\mu g \cdot mL^{-1}$ in 100 mM $NaHCO_3$-buffered saline [pH 8.2]). Unincorporated FITC-avidin was removed by washing with cold buffered saline. Filters were cut out and mounted on glass slides with PBS/glycerol (Citifluor, Ltd.) for viewing by epifluorescence microscopy.

RESULTS

Paraphysomonas *species distinctions. Paraphysomonas imperforata, P. bandaiensis,* and *P. vestita* have been described as three closely related species of *Paraphysomonas* based on morphological similarity of the siliceous scales they produce (Preisig and Hibberd 1982b). All three species produce scales with round, solid, plate-like bases bearing a single imperforate spine (Fig. 1A–E). Distinctions between the scales produced by these three species include the presence or absence of a thickened rim at the edge of the scale, spine length, spine thickness, spine termination, and the ratio of spine length to base width. These differences are subtle enough that they possibly may be subspecies (Preisig and Hibberd 1982b), although all three species descriptions are accepted at this time. *Paraphysomonas foraminifera* has been described as a species closely related to the triumvirate of *P. imperforata, P. bandaiensis,* and *P. vestita* based on the similarity of its scales, but it has been placed in a separate subgroup from the latter three species because it produces a meshwork scale base rather than a solid base (Preisig and Hibberd 1982b). In contrast to these species, *P. butcheri* produces plate-like and crown-like scales (without a spine) that are composed of a meshwork structure (Fig. 1F).

Phylogenetic analyses of Paraphysomonas species. A global phylogenetic analysis of the sequence data for the five *Paraphysomonas* species examined in this study confirmed the chrysophyte heritage of this genus (Fig. 2). All five of these species branched consistently with other chrysophyte taxa included in our analyses. These chrysophyte taxa (including *Paraphysomonas*) in turn branched together with the synurophyte algae. This result is consistent with morphology-based analyses of the phylogeny of *Paraphysomonas,* as well as recent DNA sequence information (Sandgren et al. 1995, Rice et al. 1997a, Saunders et al. 1997).

The genus *Paraphysomonas* appears to be monophyletic based on our analyses of sequence information (Figs. 2, 3). Monophyly was well supported for four of the five species examined in this study (*P. vestita, P. imperforata, P. foraminifera,* and *P. ban-*

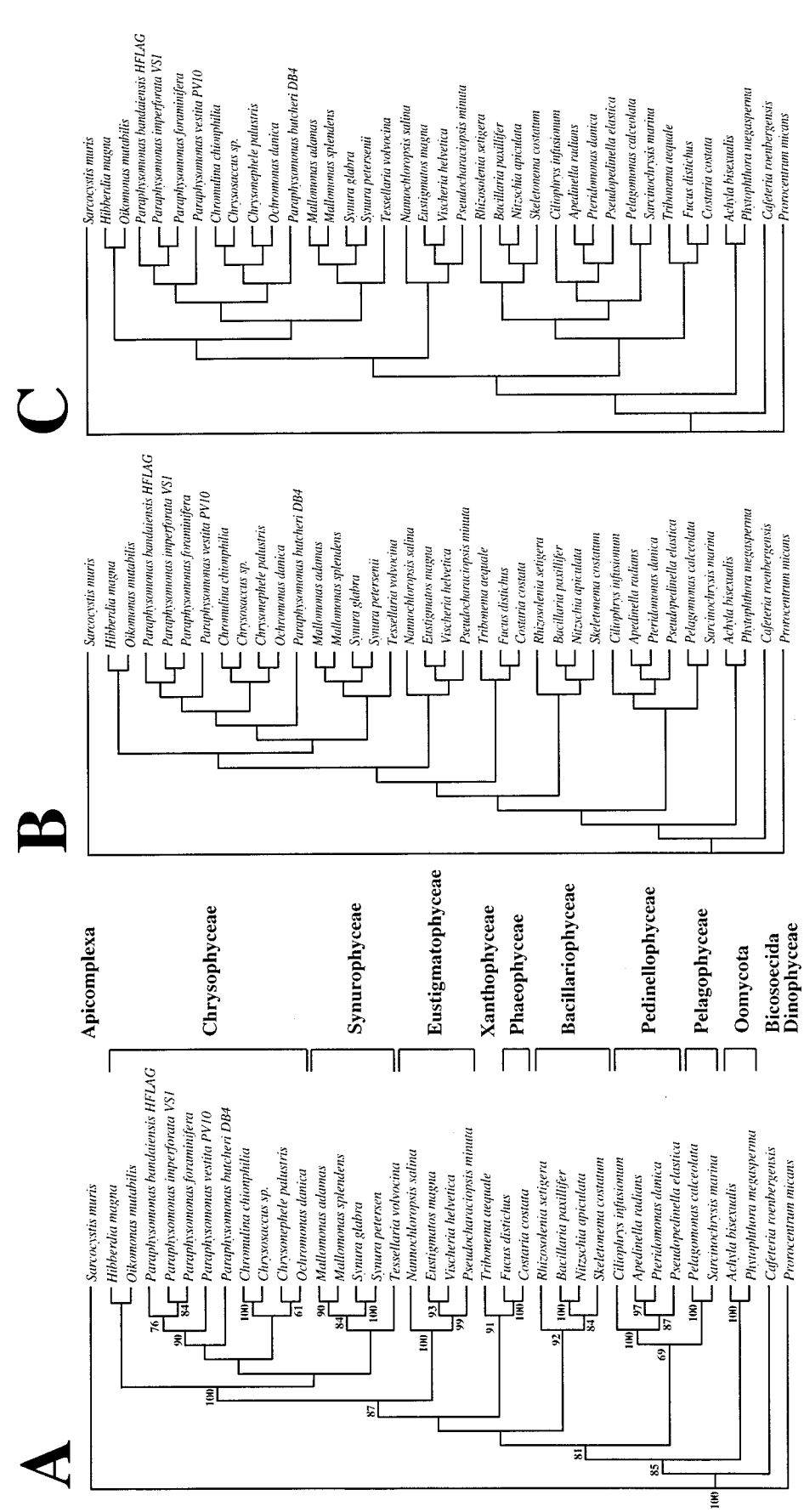

FIG. 2. Phylogenetic analysis of five species of *Paraphysomonas* and 33 other protistan species representative of closely and distantly related taxa. The trees were generated by maximum likelihood analyses (see Materials and Methods for further description of parameters) in order to obtain a global perspective on the ribosomal phylogeny of the genus *Paraphysomonas*. These three equally likely trees (A, B, C) differ in the placement of *P. butcheri*. Bootstrap analysis of the same data set shows a lack of support for the branch order within the chrysophytes.

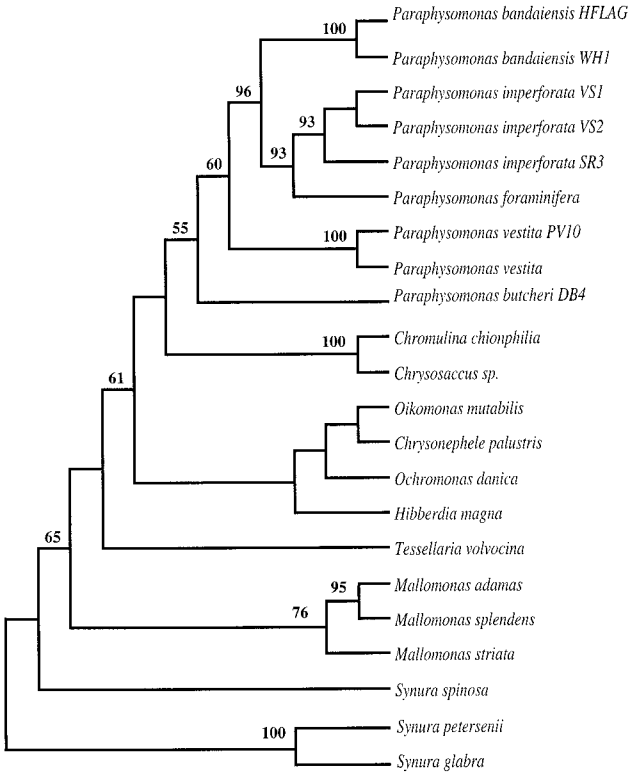

FIG. 3. Phylogenetic analysis of five species of *Paraphysomonas* and 13 other chrysophyte/synurophyte species. This tree was generated by maximum likelihood analysis.

daiensis). *Paraphysomonas vestita* in these analyses diverged prior to *P. imperforata, P. foraminifera,* and *P. bandaiensis* in both the global phylogeny (Fig. 2) as well as in a more focused phylogenetic analysis (Fig. 3). Our analyses also indicated that *P. butcheri* branched with, and was ancestral to, the latter four *Paraphysomonas* species, although with a low bootstrap value (<50% in the global analysis and 55% in the restricted analysis).

Intraspecific sequence dissimilarity was present for all three species (*P. imperforata, P. bandaiensis, P. vestita*) for which multiple isolates were sequenced. We sequenced only one isolate in the case of *P. vestita* (PV10) but full-length SSU rDNA sequence information for another isolate was available through GenBank. In the cases of *P. imperforata* and *P. bandaiensis,* there were few intraspecific sequence differences. Of the five identified sequence differences among the three *P. imperforata* isolates, one base change was unique to strain VS2, two indels were unique to isolate SR3, one indel was shared by VS2 and SR3, and one was shared by VS1 and VS2. There were only two indels among the two *P. bandaiensis* isolates. This latter amount of sequence dissimilarity is very small, and although it may actually represent differences among isolates, it could also be a consequence of sequencing error.

Comparison of the two *P. vestita* sequences showed considerable intraspecific sequence dissimilarity. There were 44 alignable base changes within the SSU rDNA of the two *P. vestita* isolates, in addition to three A/T-rich insertions that were not present in any of the other *Paraphysomonas* species examined in this study. The insertions were not identical between the two *P. vestita* strains, but they did occur at the same positions in the sequence. The *P. vestita* sequences were also quite distinct from the sequences of *P. bandaiensis, P. imperforata,* and *P. foraminifera,* even when the A/T-rich inserts were removed from the analyses. This finding was particularly unexpected given the strong morphological similarity between scales produced by these four species.

Probe design, testing, and application. Sequence comparisons of the *Paraphysomonas* species and closely related species were used to design several oligonucleotides that would hybridize with variable regions of the SSU rRNA of *P. bandaiensis* and *P. imperforata.* One probe was also designed to target both of these species as well as *P. vestita* (Table 2). Probes were designed to avoid intraspecific sequence variability in cases where multiple strains of a species were sequenced. The four probes designed to target *P. bandaiensis* (Pband 635, Pband 663, Pband 1683, Pband 706) possessed three or more mismatches with all other *Paraphysomonas* species and with other sequences searched through GenBank. Three probes designed to target *P. imperforata* (Pimp 635, Pimp 663, Pimp 1683) also possessed three or more mismatches against all sequences except for *P. foraminifera.* Each of the three probes designed to target *P. imperforata* had one base mismatch for *P. foraminifera.* The probe designed to target *P. bandaiensis, P. imperforata,* and *P. vestita* (Pbiv 1537) also matched *P. foraminifera* perfectly but possessed three mismatches with the remaining *Paraphysomonas* species examined in this study (*P. butcheri*), and three or more mismatches with other sequences in the database.

Dissociation temperatures for each of the probes were determined empirically (Table 2), and these values were used to estimate optimal conditions for probe application. Probes were then tested against target and nontarget DNA in a slot blot format (Fig. 4). The relative quantity of DNA in each slot was determined by hybridization with a eukaryote-specific probe (Fig. 4, Euk 1209). The weak reaction at location 2d on this blot indicates that less DNA for this strain was loaded on the membrane than for other species. The unprobed eubacterial DNA (location 5h) was a negative control and indicated no cross-reactivity of any of the probes with bacterial DNA.

The Pbiv 1537 probe designed to target the three closely related *Paraphysomonas* species in our culture collection (*P. vestita, P. bandaiensis, P. imperforata*) hybridized with all three target species tested in this study (locations 1a–2d in Fig. 4) but had virtually no cross-reactivity with DNA from other species tested

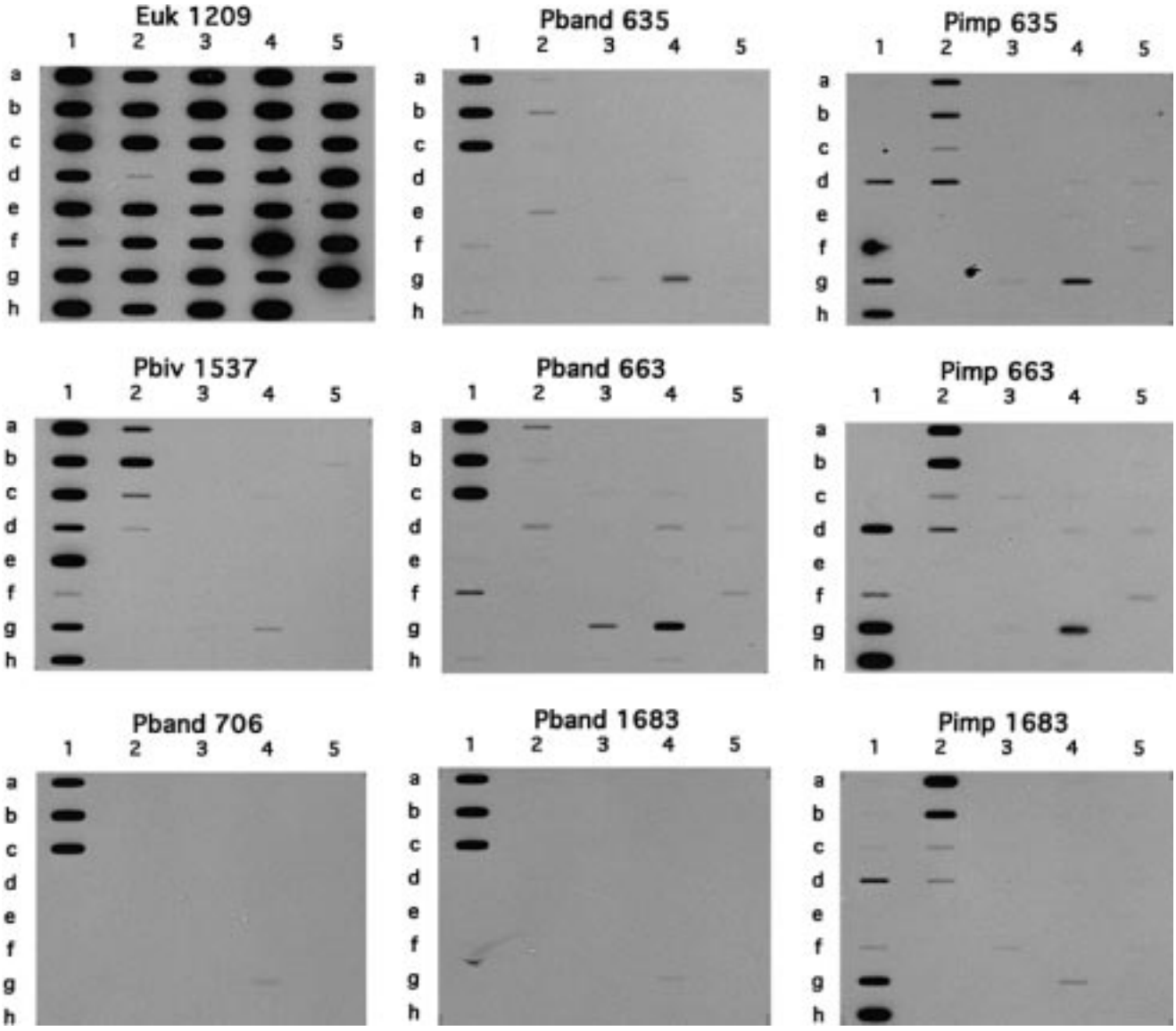

FIG. 4. Slot-blot hybridizations of ^{32}P-labeled rDNA probes against total extracted nucleic acids from a variety of protistan species and one bacterial strain. The protistan species tested included strains of *Paraphysomonas*; other phototrophic and heterotrophic chrysophytes; several nonchrysophyte algal taxa representing the Synurophyceae, Bacillariophyceae, Prymnesiophyceae, Prasinophyceae, Chlorophyceae, Dinophyceae, Eustigmatophyceae, Dictyochophyceae, and Rhodophyceae; two bicosoecids; a kinetoplastid; and a ciliate protozoan (clone designations are in parentheses). Dark bands indicate strong hybridizations. Locations of the nucleic acids for each species are given by column (number) and row (letter): 1a, *P. bandaiensis* (WH1); 1b, *P. bandaiensis* (Hflag); 1c, *P. bandaiensis* (SS); 1d, *P. imperforata* (CK4); 1e, *P. vestita* (PV10); 1f, *P. vestita* (DB1); 1g, *P. imperforata* (VS1); 1h, *P. imperforata* (JE1); 2a, *P. imperforata* (WB1); 2b, *P. imperforata* (CK3); 2c, *P. imperforata* (CK2); 2d, *P. imperforata* (SR3); 2e, *P. butcheri* (DB4); 2f, unidentified heterotrophic chrysomonad (SR1); 2g, *Spumella* sp. (EP1); 2h, *Ochromonas tuberculata*; 3a, *Chromulina chionophilia*; 3b, *Dinobryon cylindricum* (Dino13); 3c, *Pseudopedinella pyriformis* (Chryso-f); 3d, *Eustigmatos magna*; 3e, *Chrysolepidomonas dendrolepidota*; 3f, *Mallomonas caudata*; 3g, *Synura petersenii*; 3h, *Chrysochromulina ericina*; 4a, *Emiliania huxleyi*; 4b, *Skeletonema* sp.; 4c, *Thalassiosira weissflogii*; 4d, *Amphidinium* sp.; 4e, *Chlorella* sp. (993); 4f, *Dunaliella tertiolecta*; 4g, *Euglena gracilis*; 4h, *Olisthodiscus luteus*; 5a, *Porphyridium* sp.; 5b, unidentified prasinophyte (Prasino-O); 5c, *Cafeteria roenbergensis* (Cflag); 5d, *Cafeteria* sp. (SR4); 5e, unidentified heterotrophic flagellate (CK1); 5f, *Bodo parvulus* (Bodo); 5g, *Uronema* sp. (Bbcil); 5h, *Cytophaga johnsonii*. For each probe shown, the following hybridization (H) and wash (W) temperatures (° C) were employed: EUK 1209, 37 (H) 37 (W); PBIV 1537, 37 (H) 38 (W); PBAND 635, 45 (H) 53 (W); PBAND 663, 38 (H) 45 (W); PBAND 706, 45 (H) 45 (W); PBAND 1683, 45 (H) 45 (W); PIMP 635, 38 (H) 45 (W); PIMP 663, 45 (H) 53 (W); PIMP 1683, 45 (H) 45 (W).

in these blots, including the nontarget *Paraphysomonas* species *P. butcheri*. *Paraphysomonas foraminifera* was not available for our study, so hybridization of Pbiv 1537 with that species could not be examined. However, sequence comparison (with the sequence available through GenBank) indicated a perfect match for that species. Therefore, Pbiv 1537 presumably would be effective for *P. foraminifera*.

Probes Pband 706 and Pband 1683 were effective in probing the target species *P. bandaiensis* with virtually no cross-reactivity with nontarget species (including the other *Paraphysomonas* species tested).

Probes Pband 635 and Pband 663 both hybridized strongly with DNA from the target species *P. bandaiensis*. Some cross-reactivity for Pband 635 and Pband 663 was noted with *Euglena gracilis* (location 4g), and a very weak cross-reactivity was apparent with a few of the nontarget *Paraphysomonas* strains.

Probes Pimp 635, Pimp 663, and Pimp 1683 all hybridized strongly with the target species (*P. imperforata*). Some cross-reactivity was again noted for these probes with *E. gracilis,* and very weak cross-reactivities were noted with one strain of *P. vestita* for Pimp 663 and Pimp 1683. All cross-reactions were minor compared with reactivities to target DNA, except for *E. gracilis*. Cross-reactivities of the various probes with *E. gracilis* in these blots were puzzling because they were not caused by sequence similarity. Interestingly, we have not been able to probe *E. gracilis* in whole-cell hybridizations with the use of eukaryote-specific oligonucleotides or our probes designed for species of *Paraphysomonas*. Our method has been designed specifically to be used in quantitative *in situ* hybridizations. Cross-reactions are of little importance if they do not produce false positives when applied in that format. Moreover, the size and shape of *E. gracilis* is sufficiently distinct that it would be difficult to confuse it with our *Paraphysomonas* species. Therefore, the cross-reactivity of our probes to *E. gracilis* in our slot blots is probably of no consequence.

Hybridization of our oligonucleotide probes to extracted DNA demonstrated the ability of the probes to recognize target sequences, but these reactions do not necessarily indicate the effectiveness with which the probes can be applied to visualize target species in mixed assemblages of protists. Our goal in designing and applying these oligonucleotide probes was to develop a method that could be used to detect, identify, and quantify intact protistan cells in natural water samples. For this reason, we conducted studies with cultures of *Paraphysomonas* species to optimize the use of our probes for identifying these species in natural water samples and cultures.

Probes designed to target *P. imperforata* or *P. bandaiensis* were tested on formalin-preserved cells harvested at various stages of the population growth cycle. These two species represent the most closely related species of *Paraphysomonas* that were available during this study and were, therefore, the most appropriate test of probe specificities. Three-probe hybridizations demonstrated high species specificity against the various strains in our culture collection (Fig. 5). Probes Pimp 635, Pimp 663, and Pimp 1683, which targeted *P. imperforata,* resulted in brightly fluorescent target cells (arrows in Fig. 5A). Nontarget *P. bandaiensis* cells in this preparation were barely visible by their weak background fluorescence. Similarly, probes Pband 635, Pband 663, and Pband 1683, designed for *P. bandaiensis,* produced a strong fluorescent signal in the target spe-

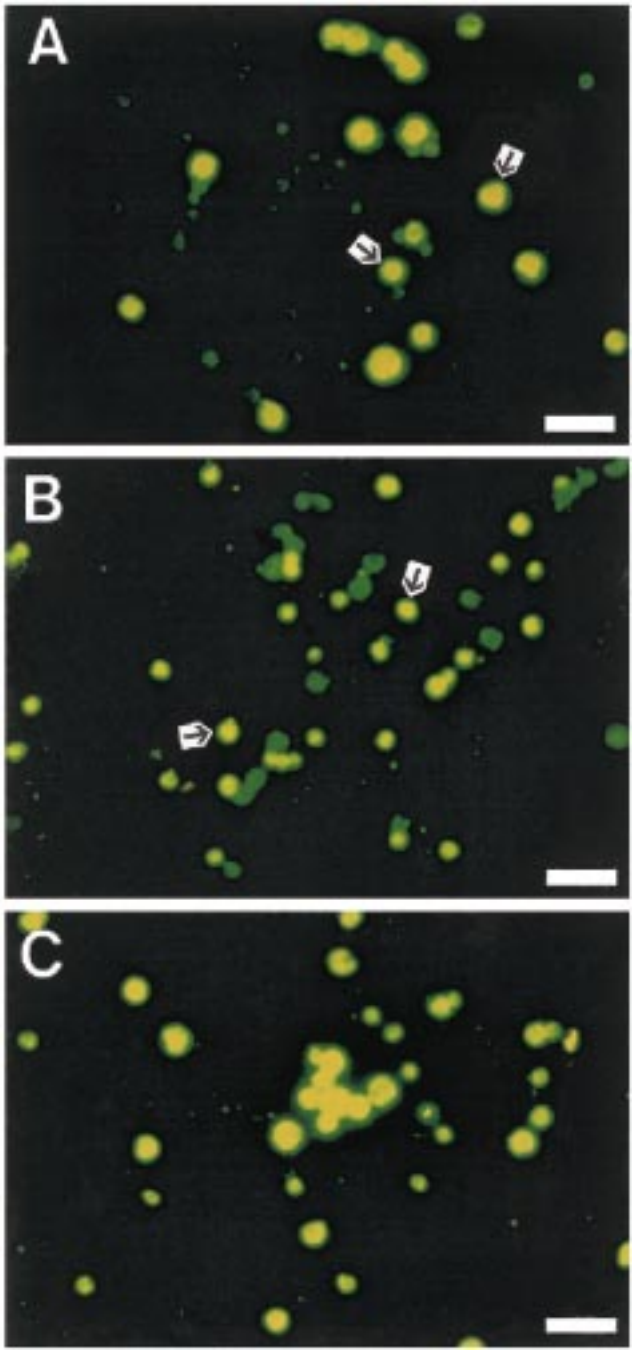

Fig. 5. Whole-cell hybridizations of a mixed culture of *P. imperforata* and *P. bandaiensis* using three probes (Pimp 635, Pimp 663, Pimp 1683) that target *P. imperforata* (A), three probes (Pband 635, Pband 663, Pband 1683) that target *P. bandaiensis* (B), or three eukaryote-specific probes (C). The larger *P. imperforata* cells (arrows in A) were highly fluorescent when *P. imperforata*-specific probes were employed, whereas the smaller *P. bandaiensis* cells displayed only a small amount of background fluorescence. The smaller *P. bandaiensis* cells (arrows in B) fluoresced brightly when probes specific to *P. bandaiensis* were used, whereas the larger *P. imperforata* cells displayed only background fluorescence. Cells from both species probed efficiently when eukaryote-specific probes were used (C). Marker bar = 20 μm.

cies (arrows in Fig. 5B), whereas the nontarget *P. imperforata* cells in this preparation were barely visible by their background fluorescence. Both species in this mixed culture were readily hybridized by eukaryote-specific probes and were clearly visible (Fig. 5C).

DISCUSSION

Phylogeny of Paraphyosomonas. The heterotrophic genus *Paraphysomonas* has been firmly established as a ''colorless'' chrysophyte taxon based on ultrastructural features of the cell (Kristiansen and Andersen 1986, Sandgren et al. 1995). Recently, rDNA sequence information has been reported that supports this traditional/historical classification (Rice et al. 1997a). Rice et al. (1997a) established the relatedness of SSU rDNA sequences for two species of *Paraphysomonas* to a larger database that included chrysophyte algae and several other protistan/algal taxa. However, complete sequence information in that study was presented only for *P. vestita* and *P. foraminifera*; therefore, analysis in that report was limited to placing these species within a larger phylogenetic context. Our analyses reported here improves our understanding of the relationships within this heterotrophic chrysophyte genus.

Our sequence information for the SSU rRNA genes confirmed the close affinity of *P. imperforata, P.bandaiensis, P. foraminifera,* and *P. vestita* that has been suggested based on scale morphology (Preisig and Hibberd 1982b). All four species produce scales with round plate-like bases and a single spine. These species branched together in our analyses with bootstrap values of 90% in the global phylogeny (Fig. 2A) and 60% in the analysis restricted to the chrysophyte/synurophyte taxa (Fig. 3). The low bootstrap value from the chrysophyte/synurophyte data set reflects the generally poor resolution of branching order within these groups based on available SSU rDNA.

Our analyses indicated a somewhat different relationship among these four closely related species, however, than the groupings proposed for these species based on scale morphology. Preisig and Hibberd (1982b) proposed that *P. imperforata, P. bandaiensis,* and *P. vestita* were more closely related to each other than to *P. foraminifera.* The latter species produce scales with plate-like bases composed of a meshwork, rather than solid plates in the former three species. Based on our results, we speculate that the divergence of *P. imperforata* and *P. foraminifera* is a recent evolutionary event. High bootstrap values (84% in the global analysis and 93% in the chrysophyte analysis) support this view. Therefore, the nature of the scale base (solid vs. meshwork) among these four species may not be as important a phylogenetic character as other features. Interestingly, Preisig and Hibberd (1982b) noted that the exact relationship among these four species was dependent on the weighting one placed on the various characters of scale morphology, and they acknowledged that a different grouping of these species would result from a different weighting of scale characters. Our molecular information has provided an independent means of examining the phylogenetic relationships among these closely related species and, consequently, a slightly altered conclusion from the one derived from the morphological data.

Sequence information for the type species of the genus (*P. vestita*) was also somewhat surprising. *Paraphysomonas vestita* showed considerable sequence dissimilarity from *P. bandaiensis, P. imperforata,* and *P. foraminifera,* a finding that is not totally consistent with the highly similar scale morphologies among these species. Comparison of homologous regions of the SSU rDNA of the *Paraphysomonas* sequences clearly separated *P. vestita* from these other three species.

Paraphysomonas vestita also was characterized by the presence of several A/T-rich insertions that were not present in any of the other *Paraphysomonas* species examined in this study and were not identical between the two *P. vestita* strains (although they occurred in the same places within the gene). Intraspecific sequence dissimilarity also was present in other regions of the SSU rRNA gene of this species. The reason for this intraspecific sequence variability is unresolved, although we speculate that it might relate to a different rate of change in the ribosomal gene of this species. A rapid mutation rate for the SSU rDNA of *P. vestita* might also explain the considerable sequence dissimilarity of this species relative to the morphologically similar species *P. bandaiensis, P. imperforata,* and *P. foraminifera.* No evidence for a pseudogene was found. At this time, we hesitate to define the two *P. vestita* isolates as different species because they do share an overall pattern of substitution. The analysis of other *P. vestita* SSU rDNA sequences should help to resolve this situation.

Comparison of the SSU rDNA sequence information for *P. vestita* and *P. bandaiensis* revealed a degree of sequence divergence that was not apparent from a comparison of their scale morphologies. The SSU rDNA sequences for *P. vestita* and *P. bandaiensis* in this study were quite distinct. The sequences for *P. vestita* were as different from the sequences for *P. bandaiensis* as they were from *P. butcheri* (the latter species being the most distantly related *Paraphysomonas* species examined in this study and possessing distinctly different scales). However, both *P. vestita* and *P. bandaiensis* possess scales that are similar in overall appearance; a solid base plate possessing a thickened rim and a single imperforate spine. Distinctions between these species include scale size, the relationship between spine length and base width, termination of the spine tip, and the size of the cell. Unfortunately, these characters are not immutable. Scale production and morphology can be affected by the concentration of silica in the wa-

ter or culture medium (Leadbeater and Barker 1995), and variability in scale morphology for these species has been noted (Preisig and Hibberd 1982a). In addition, cell size for *Paraphysomonas* species has been shown to vary with nutritional status and prey type (Fenchel 1982b, Caron et al. 1985, Goldman and Caron 1985). Based on our analyses, therefore, we suggest that SSU rDNA sequence information can provide a useful ancillary character set for distinguishing these morphologically similar species.

Our finding for the placement of *P. butcheri* outside of the other four *Paraphysomonas* species was not surprising. This species produces scales that are morphologically quite distinct from the other four species (Fig. 1F). Therefore, it was expected that *P. butcheri* might show considerable sequence divergence from the other *Paraphysomonas* species. Our analyses indicated that *P. butcheri* diverged earlier than the other *Paraphysomonas* species examined in this study, but there was only weak bootstrap support for this branching pattern (Fig. 3).

It is possible that *P. vestita* branches basally for the genus rather than *P. butcheri* (this happens in 21% of the reconstructions), but aspects of the sequence of these two species suggest that *P. butcheri* is the more likely. Foremost, *P. butcheri* possesses regions of shared sequence similarity with both the outgroup species and with *Paraphysomonas* species. In contrast, the variability in *P.vestita* is either *Paraphysomonas*-like or unique to that species (i.e. not shared with outgroup species), indicating independent evolutionary events after its evolution or very rapid rates of mutation. When the data set is limited to sites that are less ambiguous in nature (as in the global analysis), *P. vestita* falls very reliably with *P. imperforata, P. bandaiensis,* and *P. foraminifera* (Fig. 2A, 90%), whereas the position for *P. butcheri* is unresolved (Fig. 2). The addition of SSU rDNA sequences for other *Paraphysomonas* species with morphologically distinct scales might help to resolve the placement of *P. butcheri,* but the branch order within the Chrysophyceae in general is not well established by SSU rDNA. This may occur either because not enough representative genera have been examined or, more probably, because the SSU ribosomal gene is inadequate for this analysis.

Oligonucleotide probes for species of Paraphysomonas. We obtained excellent results with quantitative *in situ* hybridizations using our species-specific probes (Fig. 5). The hybridizations in Figure 5 were conducted using three probes simultaneously for each preparation that targeted either *P. imperforata* (Fig. 5A), *P. bandaiensis* (Fig. 5B), or all eukaryotes (Fig. 5C). The eukaryote-specific probes served as a positive control to demonstrate the ability of both species to be probed effectively in a single preparation. Cross reactivity was extremely low for nontarget species of protists, including other *Paraphysomonas* species, even when three probes were used in a single preparation. We used a biotin–streptavidin system for visualizing the probes in this study. Detection of cells in natural samples necessitated the use of three probes to ensure sufficient signal from target cells in poor physiological condition (Lim et al. 1993). The use of a single probe might provide sufficient signal strength as new reporter systems are developed. Therefore, the success of our triple-probe preparations in the present study indicate an acceptable result even in a worst case scenario.

We tested our probes on the most closely related species of *Paraphysomonas* available to us. In order to guarantee true species specificity, however, it will be necessary to continue to test the probes against newly isolated specimens of both target and nontarget species of the genus. It is necessary to establish that probes will hybridize with all strains of the target species and not with nontarget species in order to constitute a useful tool for ecological studies of these species. Obviously, this goal cannot be attained in a single study but rather is an ongoing effort, and it is a necessity for any probe applied to natural communities of organisms. There are approximately 50 species of *Paraphysomonas* that have been described up to the present time (Preisig and Hibberd 1983, Wujek and Saha 1995), and we have tested the specificity of our probes only against four of these species. Whereas similarity in scale morphology would indicate that we have used some of the most appropriate species for this work (i.e. most closely related species), empirical verification of our results with new isolates is highly desirable.

This study expands the number of species of *Paraphysomonas* for which oligonucleotide probes are now available. The design and application of several oligonucleotide probes for the freshwater species *P. vestita* have been reported previously (Rice et al. 1997a, b). Collectively, these three species represent some of the most commonly encountered and widely distributed species of this genus. The existence of probes that can identify these species within mixed, natural assemblages of nanoplankton should facilitate ecological and biogeographical studies of these taxa.

Identification of protists in natural water samples. Ideally, methodologies for identifying and enumerating small protists in natural water samples should be relatively rapid and quantitative. They also must be able to identify accurately species that display considerable morphological variability. Significant morphological variability (i.e. variability that might confound identification) is well known among microalgal taxa, as well as flagellated and amoeboid protozoa, as a consequence of environmental conditions or life cycle events (Fenchel 1982a, Elbrächter et al. 1987, Petrushevskaya and Swanberg 1990). In some cases the number of these morphological transformations can be extreme (Burkholder and Glasgow 1997), and confusion regarding the taxonomic usefulness of some morphological characters

has led to such disparate problems as the description of synonymous species (Cedhagen and Tendal 1989) or the classification of protists into paraphyletic groupings (Hinkle and Sogin 1993).

We have previously reported the development and application of a whole cell hybridization method for visualizing and counting individual protistan cells within the genus *Paraphysomonas* (Lim et al. 1996). Our method relies on the use of Transwell cell culture inserts (Costar) for tissue culture dishes to quantitatively retain the protistan assemblage during these procedures (Lim et al. 1996). As a result, it is now feasible to obtain quantitative information on the abundance and distribution of nanoplanktonic protists in natural samples. Combined with the use of eukaryote-specific probes, or the species-specific probes described and tested in this report, this approach has proven effective for enumerating total nanoplankton (Lim et al. 1996) as well as establishing the abundances of individual species within mixed natural assemblages of protists (Lim 1997, Lim et al. 1999). These developments represent significant advances in our ability to study the ecology of individual species of small protists in nature. These tools will gradually improve and expand as sequence databases for small protists are augmented and as these databases are exploited for probe design and application.

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