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INTER- AND INTRASPECIFIC GENETIC VARIATION IN TWELVE *PRYMNESIUM* (HAPTOPHYCEAE) CLONES[1]

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ABSTRACT

The haptophytes Prymnesium parvum *Carter and* Prymnesium patelliferum *Green, Hibberd, and Pienaar are two closely related species, which can only be distinguished by minor differences in the morphology of their organic body scales. The two* Prymnesium *species are reported to coexist at several locations, including the Sandsfjord system in southwestern Norway. Comparisons of physiology and toxicity within the two species have failed to reveal differences that can add to the small morphological distinctions used to separate them. To investigate the genetic relationship between the two species, we compared the sequence of the first internal transcribed spacer region (ITS1) and length variation in one intron separating calmodulin genes for four* P. parvum *strains and eight* P. patelliferum *strains. Both the ITS1 sequence and the banding patterns obtained by PCR amplification of one intron in the calmodulin genes indicated that the* Prymnesium *isolates are related by their geographic origin instead of by their species affiliation. The results indicate that* P. parvum *and* P. patelliferum *are so closely related that they could be considered one species. Alternatively, we discuss the possibility that the two species might be joined in a heteromorphic haploid–diploid life cycle, as is now widely reported for other haptophycean algae.*

Key index words: calmodulin gene introns; haptophytes; ITS1; phylogeny; Prymnesium

Prymnesium parvum Carter and *Prymnesium patelliferum* Green, Hibberd, and Pienaar are two closely related species of the genus *Prymnesium* Conrad (Prymnesiophyceae, Haptophyta). They are identical when viewed by light microscopy (LM), and can only be distinguished by minor differences in the morphology of the organic body scales external to the plasmalemma by using transmission electron microscopy (TEM) (Green et al. 1982). Both species have body scales of two different morphologies arranged in two layers. Each layer consists of only one morphological type. In *P. parvum* the scales have a radial pattern on their proximal face and concentric rings on their distal face, whereas both scale faces of both layers have a radial pattern in *P. patelliferum*. In addition the scales of *P. patelliferum* have a central thickening, which cannot be found in *P. parvum* scales. The rims of the outer scales in *P. patelliferum* are broad and upright, whereas the rim of the scales in the inner layer is broad and inflexed in *P. parvum*. The rim of the inner *P. patelliferum* scales and the outer *P. parvum* scales is narrow and inflexed (Green et al. 1982).

It has been known since 1989 that *P. parvum* and *P. patelliferum* coexist in annual blooms in fjord arms in Ryfylke, southwestern (SW) Norway (Edvardsen and Paasche 1997, and references therein). In addition they are found to cooccur in a fish pond near Büsum, northern Germany (Urban Tillmann, pers. commun.), and scales of the two species have been found together in the same water samples from the Baltic Sea (Helge A. Thomsen, pers. commun.). *Prymnesium parvum* is known to be ichthyotoxic, whereas *P. patelliferum* is only assumed to be so (e.g. Yariv and Hestrin 1961, Ulitzur and Shilo 1964, Shilo 1971, Larsen et al. 1993a, Moestrup 1994). Both species thus represent a constant threat to fish farmers wherever they occur and bloom.

Growth and toxicity in *P. parvum* has been extensively studied (e.g. Yariv and Hestrin 1961, Ulitzur and Shilo 1964, Padilla 1970, Shilo 1971, Holdway et al. 1978, Brand 1984), and some of the first reports of growth and toxicity in *P. patelliferum* suggested that this species might grow faster and be less toxic than *P. parvum* (Arlstad 1991, Meldahl et al. 1994). Toxicity of *P. patelliferum* has been tested against other algae (Arlstad 1991) or against blood and nerve cell preparations (Meldahl et al. 1994). Studies in which three *Prymnesium* clones were included (one of *P. parvum* and two of *P. patelliferum*), however, have shown that intraspecific differences in growth rates as well as toxicity (as measured against *Artemia* nauplii) might be just as great as interspecific differences between the two *Prymnesium* species (Larsen et al. 1993a).

A comparison of the 18S small subunit ribosomal RNA (ssu-rRNA) gene sequence from *P. patelliferum* isolated from the English Channel (Cavalier-Smith 1993) and *P. parvum* isolated from Denmark (Medlin et al. 1998) showed that only two nucleotide differences separate the two species. In contrast, 27 base pair (bp) differences are found between *P. par-*

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TABLE 1. *Algal strains used in this study.*

Species	Origin	Culture collection	Culture code	Isolated by, year
Prymnesium parvum	Flade Sø, Thy, Denmark	Scandinavian, Copenhagen	K0081	T. Christensen 1985
P. parvum	Lovrafjord, Ryfylke, Norway	Bergen	RL10parv93	A. Larsen 1993
P. parvum	Lovrafjord, Ryfylke, Norway	Bergen	RL9parv93	A. Larsen 1993
P. parvum	R. Blackwater, England	Plymouth	PCC94	D. R. W. Butcher 1952
P. patelliferum	Hylsfjord, Ryfylke, Norway	Oslo	RHpat89	E. Paasche 1989
P. patelliferum	Hylsfjord, Ryfylke, Norway	Bergen	RHpat93	A. Larsen 1993
P. patelliferum	Lovrafjord, Ryfylke, Norway	Bergen	RLpat93	A. Larsen 1993
P. patelliferum	Hylsfjord, Ryfylke, Norway	Bergen	RHT1pat94	A. Larsen 1994
P. patelliferum	Lovrafjord, Ryfylke, Norway	Bergen	RLK6pat94	A. Larsen 1994
P. patelliferum	Sandsfjord, Ryfylke, Norway	Bergen	RS2pat94	A. Larsen 1994
P. patelliferum	Norman Bay, Australia	Scandinavian, Copenhagen	K0252	Ø. Moestrup 1987
P. patelliferum	The Fleet, Dorset, England	Plymouth	PCC527	D. Hibberd 1976
Chrysochromulina polylepis	Skagerrak, Norway	Oslo	K	B. Edvardsen 1988

vum/P. patelliferum and *Prymnesium calathiferum* Chang and Ryan (Medlin et al. 1998). Thus, molecular data in addition to morphology, growth, and toxicity indicate that *P. parvum* and *P. patelliferum* are closely related.

Although the 18S rDNA can be used to delineate species of the same genus (e.g. Medlin et al. 1991, Buchheim and Chapman 1992, Bakker et al. 1994, Bird et al. 1994, McNally et al. 1994, Medlin et al. 1994, Friedl 1995, Gast and Caron 1996), other regions of the genome can be used to obtain more information about the genetic variation between and within the species if there is little or no resolution in the 18S rRNA gene. Noncoding spacer regions of the DNA, such as nuclear ribosomal DNA internal transcribed spacers (ITS) and the spacer region between the plastid-encoded small and large RUBISCO subunits, evolve at a faster rate and show greater variability than coding DNA. At lower taxonomic levels or between recently evolved species, these regions have been used routinely for studying genetic differences at species and subspecies levels among the algae and other organisms (e.g. Destombe and Douglas 1991, Bakker et al. 1992, Kooistra et al. 1992, Maggs et al. 1992, Goff et al. 1994, van Oppen et al. 1995). The RUBISCO spacer region may be more conserved in algae than the ITS regions (Goff et al. 1994, Lange 1997).

More recently, other noncoding regions have been investigated for their usefulness in species and strain identification, especially in species that have evolved recently. The calmodulin genes occur in an operon and contain a series of introns that can be used as neutral markers to assay species at the population level (Côrte-Real et al. 1994). PCR amplification of these introns, using primers based on conserved exon sequences, can detect intraspecific DNA polymorphism in the genome. The resulting fragment pattern, in which different band lengths represent different alleles, thus provides a tool for delineating strains or populations of a species.

To investigate more fully the genetic relationships among different strains of *P. parvum* and *P. patelliferum*, we compared the sequence of the first internal transcribed spacer region (ITS1) in the ribosomal operon and the length variation in the third intron separating the calmodulin gene (*CaM-I*). *Prymnesium parvum* and *P. patelliferum* isolated during blooms in the Ryfylke fjords, SW Norway, in 1989, 1993, and 1994 were investigated, along with *P. parvum* strains from England and Denmark and *P. patelliferum* strains from Australia and England.

MATERIALS AND METHODS

Cultures and DNA extraction. The algal strains used in this study are listed in Table 1. Species designations were confirmed by means of TEM for all strains. Stock cultures were grown in tubes containing 10 mL filtered (0.45-μm cellulose nitrate filters, Sartorius, Goettingen, Germany) seawater diluted to 8 practical salinity units (psu). Vitamins, chelated trace metals, and nutrients were added as in IMR ½ medium (Eppley et al. 1967). The stock cultures were grown at 15° C under white fluorescent light with a photon fluence rate (PFR) of 150 μmol photons·m^{-2}·s^{-1} and a 14:10 h LD cycle. The cultures used for DNA extractions were grown under the same conditions as the stock cultures, in Erlenmeyer flasks containing 5 L of medium.

Cultures were harvested during exponential growth phase by filtration onto 3-μm polycarbonate filters (Nuclepore, Pleasanton, California). The filters were frozen immediately and kept at −70° C until their DNA was extracted. The DNA extractions were performed using a 3% (w/v) CTAB procedure as described in Doyle and Doyle (1990).

PCR amplification of the ITS region. The sequences of the primers used in our PCRs are listed in Table 2. Primer combinations used to amplify the ITS region were 1400F+ITS2R, 1400F+DICR, 1055F+DICR, and 1055F+639R (Table 2). One of the primers in each combination was biotinylated at its 5′ end to facilitate solid phase sequencing (DYNAL, A.S., Oslo, Norway). In some instances it was necessary first to perform a PCR with nonbiotinylated primers, purify the PCR product with the GlassMax DNA Isolation Spin Cartridge System (Gibco BRL, Life Technologies, Inc., Gaithersburg, Maryland) and then use it as a template in a PCR with biotinylated primers.

The PCR mixtures (100 μL) contained 100–400 ng total nucleic acids, 3 pmol of each primer, 10 nmol dNTPs, 10 μL 10× PCR buffer with 2 μM $MgCl_2$, and 1 U (Super Taq, H. T. Biotech) or 2 U (Ampli Taq Cetus, Perkin Elmer, Branchburg, New Jersey) enzyme. Product yield was improved by adding 0.5 μL T4-gene-32 protein (Amersham, Buckinghamshire, United Kingdom) or 10 μL 50% acetamide (Sigma Chemical Co., St. Louis, Missouri). Double-stranded amplification of the ITS region was performed with an initial denaturation step of 97° C for 6 min followed by 30 cycles of either cycle 1 (94° C for 2 min, 2 min ramp down to 37° C, 2 min at 37° C, 3 min ramp up to 72° C, 6 min at 72° C) or cycle 2 (94° C for 2 min, 45° C for 2 min, 72° C for 4 min);

TABLE 2. *Oligonucleotide primers used for PCR and sequencing, Sd synthesis direction: F, forward; R, reverse.*

Code	Sd	Nucleotide sequence 5′ to 3′	Position
1055F	F	GGTGGTGCATGGCCG	1269 in 18S
1400F	F	TGT/CACACACCGCCCGTC	1629 in 18S
ITS2R	R	GCTGCGTTCTTCATCGATGC	24 in 5.8S
DICR	R	ACTCTCTTTTCAAAGTCCTT	370 in 28S
639R	R	GGTCCGTGTTTCAAGACGGG	710 in 28S
CAD1	F	CCGAATTCCAAGAC/TATGATXAAC/TGAA/GGT	calmodulin gene
CAD3	F	GGACAGAATTCXACXGAA/GGCXGA	calmodulin gene
CAD2	R	CCGAATTCATCTTXCG/TXGCCATCAT	calmodulin gene

both cycles were followed by an extension step of 72° C for 9 min. The amplification products were checked for correct length, purity, and yield on ethidium-bromide-stained 0.75% agarose gels (Sambrook et al. 1989), visualized by ultraviolet (UV) illumination, and photographed with Polaroid 667 film.

Sequencing of the ITS1 region. Single-stranded DNA (ssDNA) was obtained by binding amplification products onto Streptavidin-coated magnetic M-280 Dynabeads following a slightly modified protocol of DYNAL (Lange 1997). The ssDNA products were used as template in dideoxynucleotide chain termination sequencing reactions (Sanger et al. 1977) of both coding and noncoding strands using either the T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden) or the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio). Sequencing primers were used at concentrations of 100 ng·μL^{-1}. The 1400F primer was used for generating sequence data for the forward strand and the ITS2R for the opposite strand (Table 2). Additional sequencing reactions with dITP substituted for dGTP in the termination reactions eliminated gel artifacts, such as compressions.

Sequence alignment and phylogenetic analyses. The sequences were aligned on a VXT 2000+ (Digital, U.S.A.) using the Olsen sequence editor (Larsen et al. 1993b). The *Prymnesium* sequences were aligned with the ITS sequence of *Chrysochromulina polylepis* Manton and Parke. The nuclear-encoded small subunit rRNA gene from *C. polylepis* differs by only 23 bp from those of the two *Prymnesium* species (Medlin et al. 1998); therefore, this species was used as an outgroup.

The sequences were analyzed using both maximum parsimony and distance matrix methods. Maximum parsimony analysis was performed using PAUP (version 3.1.1, Swofford 1993) with the heuristic search invoking the TBR branch-swapping algorithm and the MULPARS option. The distance analysis was executed with PHYLIP (version 3.5e, Felsenstein 1993) using the Kimura model (Kimura 1980), which allows for a difference between transversion and transition rates in the base substitution. The neighbor-joining method (Saitou and Nei 1987) was used to infer a tree from the Kimura distance matrix. Bootstrap analysis using a 50% majority rule was done with 100 replicates to test the stability of the branching order.

Intron-targeted PCR of CaM-1. PCR amplification of the *CaM-1* gene was obtained by using a nested PCR. A primary PCR was performed using 150 ng nuclear DNA and the primer combination CAD3 + CAD2 (Table 2, Côrte-Real 1994). A 1.5-μL aliquot of the PCR product from this reaction was used directly as template for a second PCR using the primer combination CAD1 + CAD2 (Table 2, Côrte-Real 1994). PCR volumes were 100 μL and contained 30 pmol primers, 10 nmol dNTPs, 10 μL 10× PCR buffer with 2 μM $MgCl_2$ and 1 U enzyme (Ampli Taq Cetus, Perkin Elmer). Product yield was improved by adding 0.5 μL T4-gene-32 protein (Amersham) to the primary PCRs. The primary PCR was performed using a cycle with an initial denaturation step of 97° C for 6 min followed by 30 cycles of (94° C for 2 min, 2 min ramp down to 37° C, 2 min at 37° C, 3 min ramp up to 72° C, 6 min at 72° C) with a final extension step of 72° C for 9 min. The secondary PCRs were done under more stringent conditions: 97° C for 6 min (denaturation), 30 cycles of (94° C for 2 min, 45° C for 2 min, 72° C for 4 min), followed by an extension step of 72° C for 9 min. The amplification products were assayed on ethidium-bromide-stained 1.3% agarose gels (Sambrook et al. 1989), visualized by ultraviolet (UV) illumination, and photographed with Polaroid 667 film.

RESULTS

ITS1-sequences. Complete sequences of the entire ITS1 and stretches of the adjacent 18S rDNA and 5.8S rDNA were determined for the 12 *Prymnesium* strains and a single strain of *Chrysochromulina polylepis*. The aligned sequences are shown in Figure 1. The length of the ITS1 region of the *Prymnesium* strains varied from 255–261 bp. Boundaries of coding and noncoding regions were determined by comparison to the 18S gene of *P. parvum* and *C. polylepis* (Medlin et al. 1998) and the 5.8S rRNA gene of the chlorophyte *Chladophora albida* (Hudson) Kützing (Bakker et al. 1992). The length of the ITS1 region of *Prymnesium* is approximately the same size as the only other known haptophyte ITS1 sequences, *Phaeocystis antarctica* Karsten (259–273 bp) and *P. globosa* Scherffel (277 bp) (Lange 1997). All the *Prymnesium* sequences could easily be aligned with one another, requiring only a minimal number of insertions to maximize homology. *Chrysochromulina polylepis,* which was sequenced and included as an outgroup, possessed a considerably longer ITS1 region (430 bp) than *P. parvum* and *P. patelliferum.* The alignment of the *Prymnesium* sequences to *C. polylepis* therefore required the introduction of many insertions.

The relationship among the *Prymnesium* strains was analyzed using both distance and maximum parsimony analysis (Fig. 2). A total of 385 characters were used in the analysis (gaps were excluded). In both analyses the strains grouped by their geographical origin instead of by their species affiliation. No nucleotide differences were found between *P. parvum* strains (RL9parv93, RL10parv93) isolated from the Sandsfjord system, Norway, the *P. patelliferum* strains (RHT1pat94, RLK6pat94, RHpat93, RS2pat94, RLpat93, and RHpat89) isolated from the same fjord system and the *P. parvum* strain from Denmark (K0081). The two isolates from England (one *P. parvum,* PCC94, and one *P. patelliferum,* PCC527) were separated from one another by a single base change and differed from the "Norwegian/Danish group" by 18 bp. The absolute number of nucleotide differences between the sequence of the Australian iso-

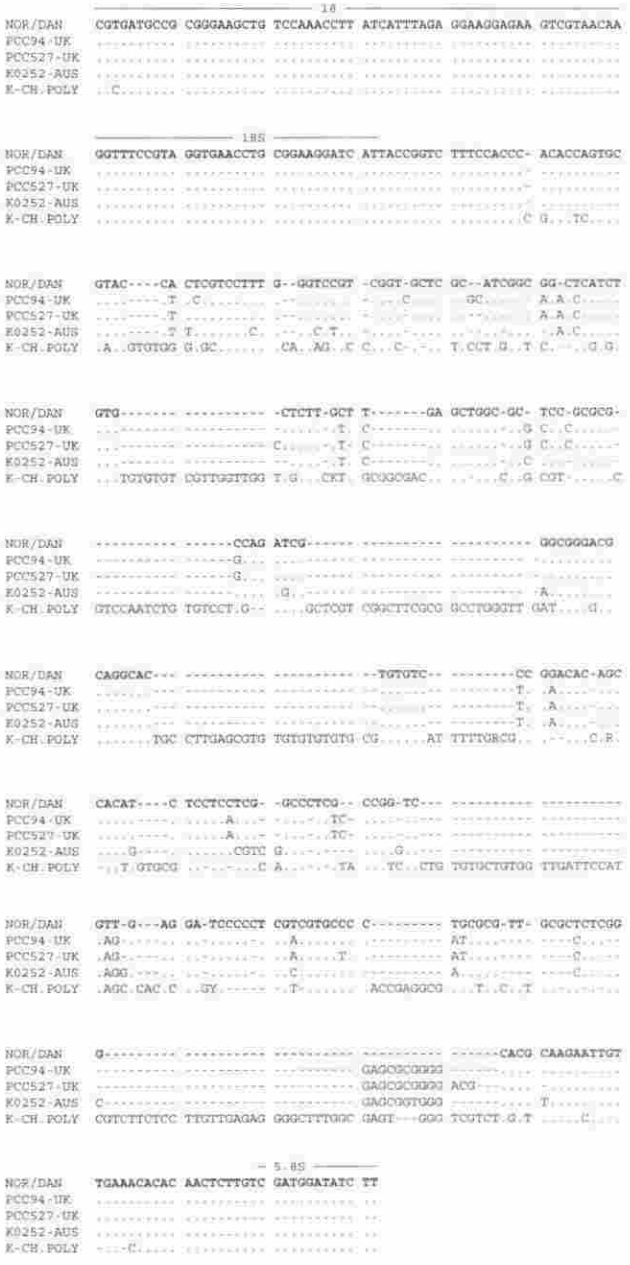

FIG. 1. Alignment of ITS1 regions with flanking coding regions from the 18S and 5.8S rRNA gene from four isolates of *Prymnesium parvum* and eight strains of *P. patelliferum* with *Chrysochromulina polylepis*. Abbreviations of the strains are as in Table 1 with a geographical notation. Nor/Dan represents the Norwegian/Danish group, which consists of the following clones, all with identical ITS1 sequence: K0081, RL10parv93, RL9parv93, RHT1pat94, RLK6pat94, RHpat93, RS2pat94, RLpat93, RHpat89. A dot indicates a position identical to the "Norwegian/Danish isolates" sequences. A hyphen is a gap introduced to improve alignment. K = G/T, R = C/T, R = A/G.

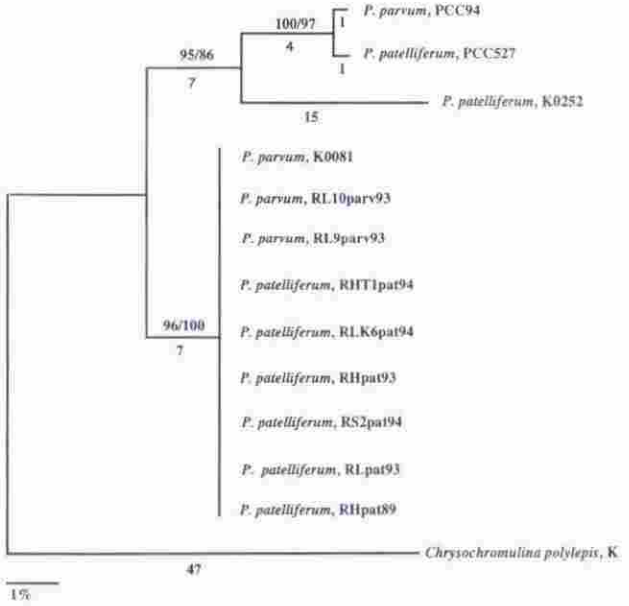

FIG. 2. Phylogenetic analysis of relationships among four strains of *Prymnesium parvum* and eight strains of *P. patelliferum* using the neighbor-joining analysis. Figures above the internal nodes are bootstrap values based on a neighbor-joining analysis (left) and on a maximum parsimony analysis (right). The absolute number of nucleotide differences between the strains are shown below the internal nodes. An isolate of *Chrysochromulina polylepis* is used as an outgroup. The distance corresponding to one change per 100 nucleotide positions is placed below the distance tree. Abbreviations of the strains are as in Table 1.

late of *P. patelliferum* (K0252) and the English strains was 20, whereas the Australian isolate differed by 29 bp from those of the Norwegian/Danish group.

Intron-targeted PCR of the calmodulin genes. Nested PCR amplification from total nucleic acid preparations from the 12 *Prymnesium* strains using the calmodulin gene primers, gave two to three major products between ~280 and 450 bp (Fig. 3). We presume that these bands represent length differences in the third intron within one calmodulin gene and thus represent different loci because the primers used in the second amplification are gene specific. Because the calmodulin gene has not been sequenced, this region may not necessarily be the third intron in these genes from *Prymnesium*. (It does, however, correspond to the third intron characterized by Côrte-Real et al. [1994].) The lengths of the amplified bands were equal (320 and 390 bp) for the Norwegian/Danish group (K0081, RL10parv93, RL9parv93, RHT1pat94, RLK6pat94, RS2pat94, RLpat93, RHpat93, and RHpat89). The band lengths of the PCR products from English and Australian strains (PCC527, PCC94, and K0252) were 320/390/450 bp, 280/320/390 bp, and 320/350 bp, respectively. The patterns derived from the PCR reactions with *P. patelliferum* and *P. parvum* from England and *P. patelliferum* from Australia thus differed from one another and from the pattern obtained from the Norwegian/Danish *Prymnesium* group.

A strain of *C. polylepis* (the K strain) has also been analyzed by nested PCR using the *CaM-1* primers. This strain is known to be haploid (Edvardsen and Vaulot 1996) and gave a single band in the analysis (Edvardsen and Medlin, unpubl.). Thus, the single

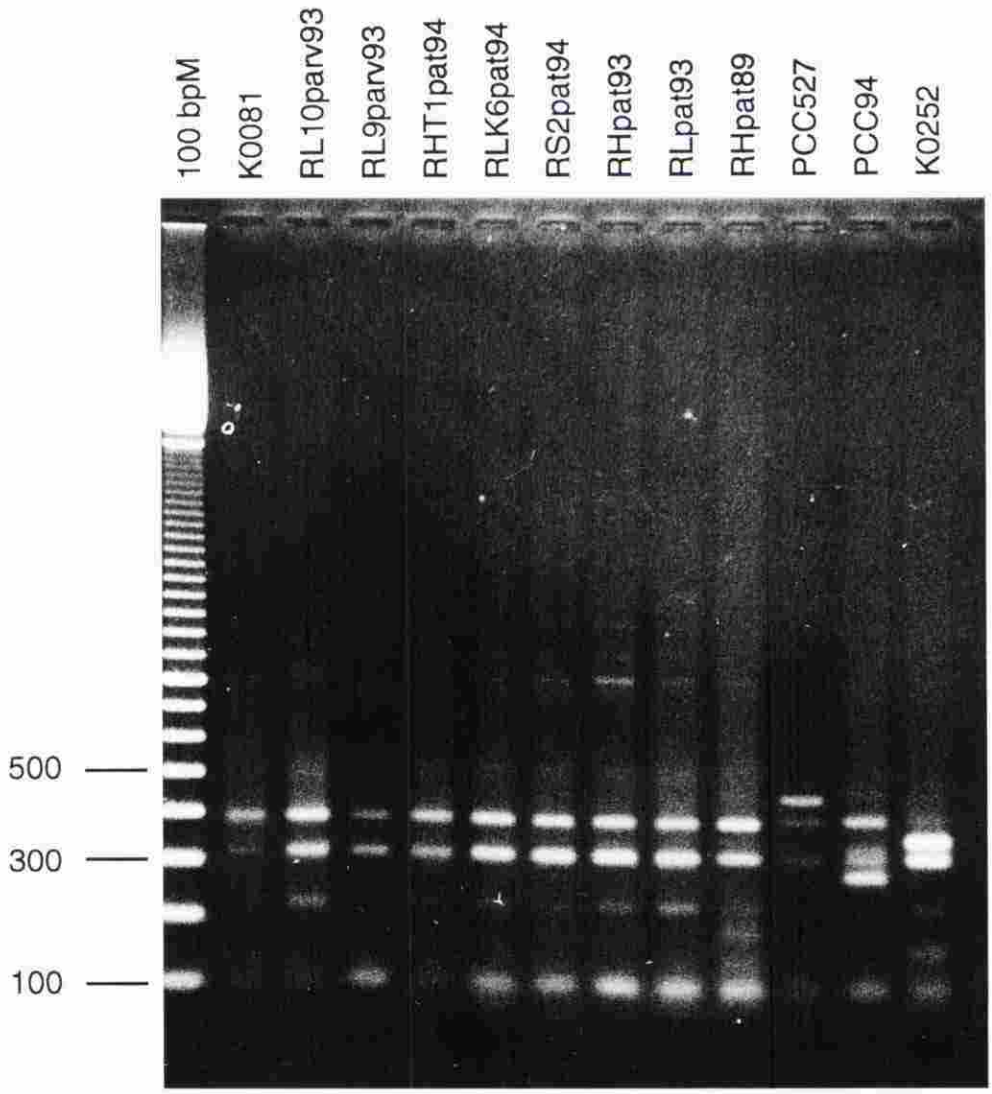

FIG. 3. Agarose gel showing band patterns obtained from *Prymnesium* isolates using calmodulin primers CAD 1, CAD 2, and CAD 3, in a nested PCR reaction. A 100-base baseladder (Pharmacia) was used as a size marker. Abbreviations of the taxa are as in Table 1.

band displayed by this species likely reflects the haploid state for this intron. In contrast, the double bands seen in the Norwegian/Danish group could be interpreted as being heterozygous and all strains with this pattern could be considered diploid. Alternatively, the gene may not be a single-copy gene in the haptophytes, and the two bands could originate from introns in different loci. The multiple bands in the PCC94 strain are more difficult to interpret without sequencing of the PCR products; however, it is possible that multiple alleles for this intron or multiple copies of the gene may occur. The determination of ploidy levels directly from the banding patterns would be possible if it were known specifically that this gene is a single-copy gene in the haptophytes.

DISCUSSION

Investigations of growth rates, toxicity, and chlorophyll content of some of the *Prymnesium parvum* and *P. patelliferum* strains used in this study have failed to reveal clear differences between the two species, although strain/population differences within each species do occur (Larsen et al. 1993a). Our molecular analyses of both ITS1 sequence data and banding patterns obtained by the PCR amplification of the third intron in a calmodulin gene clearly indicate that clones of *P. parvum* and *P. patelliferum* are related by their geographical origin. The close genetic relationship between *P. parvum* and *P. patelliferum* is an indication that they might be considered as one instead of two species.

The ITS1 sequence and the *CaM-1* band pattern in *P. parvum* isolated from Flade Sø in Denmark are identical to those found in the Norwegian *P. parvum/P. patelliferum*, and thus the Danish and the Norwegian *Prymnesium* strains likely belong to the same population. One possible explanation for this might be that the freshwater lake Flade Sø may be occasionally inundated or sprayed with seawater. Be-

cause the lake is situated very close to the western coast of Denmark, the seawater reaching the lake may contain the same algal populations as the Jutland Current reaching the southwestern coast of Norway and our sample site in the Sandsfjord system. It is therefore possible that a population of *Prymnesium* in the Jutland Current could be the same as the one blooming in the Sandsfjord system. *Prymnesium parvum* has earlier been recorded from Tjøme, Viksfjord (both southeastern Norway and Tromsø [northern Norway], Throndsen 1969), which means it might be a common part of the phytoplankton flora off the Norwegian coast. Blooms of *P. parvum* have not been recorded any other place than in the Sandsfjord system, although it may have bloomed in other places where there are no fish farms and thus may have been overlooked.

It appears that we have a situation where physiology/toxicity and genetic characters do not correspond with the morphologic characters being used to separate *P. parvum* and *P. patelliferum*. A similar situation was found among strains of the dinoflagellate *Alexandrium catenella–tamarense–fundyense* species complex (e.g. Adachi et al. 1994, 1995, 1996, Scholin and Anderson 1994, Scholin et al. 1994, 1995). Although morphological characters are the primary means of delineating different *Alexandrium* species (Balech 1985, Steidinger 1990), their validity with respect to species- and strain-level classifications has been debated (Taylor 1985, 1990). Destombe et al. (1992) pointed out a need to bring in molecular techniques in order to determine taxonomic affinities within the genus because efforts to employ biochemical characters usually have failed to indicate a high degree of congruence with conventional morphological criteria within the genus. Different regions of the genome have been used to delineate *A. tamarense* (Lebour) Balech, *A. catenella* (Whedon and Kofoid) Balech, and *A. fundyense* Balech, (Adachi et al. 1994, 1995, 1996, Scholin and Anderson 1994, Scholin et al. 1994, 1995). All results suggest that the genotypes do not correspond with the morphotype and reflect instead geographical origin of the strains. The morphological features used to define the three species have been interpreted to reflect strain-specific characters rather than clearly defined species (Adachi et al. 1994, 1995, 1996, Scholin and Anderson 1994, Scholin et al. 1994, 1995).

In contrast, a somewhat different situation is found in the haptophyte *Emiliania huxleyi* (Lohman) Hay et Mohler. The species can be divided into at least three different morphotypes: A, B, and C (Young and Westbroek 1991, van Bleijswijk et al. 1991). In this case, however, the division, based on morphological features of the coccoliths, is supported by physiological, biochemical, and immunological studies (Young and Westbroek 1991, van Bleijswijk et al. 1991). In contrast, sequences of coding (nuclear- and plastid-encoded ssu-rRNA) and noncoding (RUBISCO spacer region) parts of the genome are identical, suggesting that the separation of the morphotypes of *E. huxleyi* is too recent to be detected by base substitutions, deletions, or insertions in these coding and noncoding regions (Medlin et al. 1996). Randomly amplified polymorphic DNA (RAPD) banding patterns and genome size as measured by flow cytometry reinforce the separation of A and B morphotypes and indicate that strains within each morphotype are also related by geographical origin. Medlin et al. (1996) support Young and Westbroek's (1991) view that the A and B morphotypes of *E. huxleyi* should be recognized at a varietal level rather than at a species level and validate the following varieties: *E. huxleyi* (Lohmann) Hay and Mohler var. *huxleyi*, *E. huxleyi* var. *pujosae* (Verbeek) Young and Westbroek ex Medlin and Green, and *E. huxleyi* var. *kleijniae* Young and Westbroek ex Medlin and Green.

The results from the present study combined with results from investigations of growth rates, chlorophyll *a* content, and toxicity (Larsen et al. 1993a) can be compared with the examples of the *Alexandrium* species complex where there is a conflict between the morphological and phylogenetic species, or with the case of *E. huxleyi* where different morphotypes and genotypes have been interpreted to represent varieties rather than species. Bearing this in mind, there might be reason to ask if the recognition of *P. parvum* and *P. patelliferum* as separate species is warranted, based on the slight morphologic differences of the scale morphology. The only other *Prymnesium* species, *P. calathiferum*, for which 18S rDNA sequence data are available (Medlin et al. 1998), is genetically (and morphologically) distinct from *P. parvum* and *P. patelliferum*. In *P. calathiferum* the inner scale layer is composed of typical prymnesiophycean scales (Green et al. 1990). However, the distal face of the outer scale of *P. calathiferum* has a tall, wicker-basket-like upright rim, composed of vertical and horizontal bars (Chang and Ryan 1985) and is, thus, far more elaborate than the scales found in either *P. parvum* or *P. patelliferum*. The difference between the morphology of the cell surface scales of *P. calathiferum* and those of *P. patelliferum* and *P. parvum* can thus be characterized as more distinct than the differences in the scale morphology between the two latter species (Chang and Ryan 1985, Green et al. 1982). This could support a suggestion that *P. parvum* and *P. patelliferum* are two morphotypes of the same species rather than two distinct species. Alternatively, it may be that *P. parvum* and *P. patelliferum* have very recently speciated from one another.

However, another explanation may be equally likely. Alternation between different stages of a life cycle has been known for several years for many haptophytes (e.g. Parke and Adams 1960, von Stosch 1967). In most reported cases, the life history involves a nonmotile and a motile stage (for review see Billard 1994). However, Thomsen et al. (1991)

and Edvardsen and Vaulot (1996) have shown that both representatives of coccolithophorids and noncalcified haptophytes can be joined in haplo–diploid life cycles where all stages are motile. Furthermore, Medlin et al. (1996) reported what appear to be haploid and diploid forms in both A and B morphotypes of the nonmotile vegetative stages of *E. huxleyi*. Bearing this in mind, we think there are reasons to speculate that these two *Prymnesium* species with minor morphologic differences, identical ITS1 sequences, and identical *CaM-1* banding patterns, could be two stages in the same life cycle. Billard (1994) suggests that the organic scales surrounding the cell plasmalemma are useful phenotypic indicators of different ploidy levels in haptophytes. Documented cases of alternating diploid and haploid phases in coccolithophorids are characterized by diploid cells with organic scales having identical ornamentation on both faces of the scales and haploid cells with scales having a radial pattern on their proximal face and concentric rings on their distal face (Billard 1994). According to the hypothesis of Billard (1994) *P. patelliferum*, with a radiating pattern on both faces of its scales, could represent the diploid stage. *Prymnesium parvum*, with a radial pattern on its proximal scale face and concentric rings on the distal scale face, could be the haploid stage in a heteromorphic life cycle. It should be noted that, of all described *Prymnesium* species bearing two types of scales, *P. patelliferum* is the only one that does not have a concentric pattern at the distal face of the inner scale layer (Green et al. 1982, Billard 1983, Chang and Ryan 1985). *Prymnesium nemamethecum* (Pienaar and Birkhead 1994), which is the only other species of the genus lacking scales with a concentric pattern, is different from the rest, also, because it has three scale types (Pienaar and Birkhead 1994).

Prymnesium parvum and *P. patelliferum* are known to cooccur (Edvardsen and Paasche 1997, and references therein, Helge A. Thomsen, pers. commun., Urban Tillmann, pers. commun.), and this could suggest that they might be linked in a life cycle. Unfortunately, a change from one life cycle stage to another has never been observed in our own *Prymnesium* cultures, nor has it been reported from the literature. In contrast, the two cell types of *C. polylepis* are suggested to change from one type to the other under culture conditions (Edvardsen and Paasche 1992). Neither has there been any report of both scale types occurring on one and the same cell, as observed for several coccolithophorids now known to be linked in a haploid–diploid life cycle (Thomsen et al. 1991). The possibility of *P. parvum* and *P. patelliferum* being two motile stages in a life cycle is not excluded by this, however. Lack of changes between the two "types" in our cultures or absence of "combination cells" with both "*parvum*" and "*patelliferum*" scales could be explained by the fact that conditions in our cultures did not favor the change from one cell type to the other. Incorrect environmental conditions or absence of the right mating types in the same culture, are both examples of factors that could preclude initiation of life cycle changes. It is also possible that it has occurred, but has been overlooked.

Our results indicate that *P. parvum* and *P. patelliferum* populations isolated from the same geographical area are so closely related that we question whether we are dealing with one or two species. To find a more satisfactory explanation to this problem, further investigations of sequences of noncoding regions or applications of RAPDs could be employed. As differences in the pattern of the organic scales normally define species in the haptophycean algae, our findings are interesting in light of the general taxonomy of this group. If our findings indicate another, presently unknown, dimorphic life cycle in the haptophytes, then this information might also provide further insights into the ecology of these two important ichthyotoxic haptophytes.

An investigation into the ploidy level of the two species is presently underway to determine if *P. parvum* and *P. patelliferum* are two stages of a heteromorphic life cycle. Preliminary results from this investigation show that the DNA content of *P. parvum* is not identical to that of *P. patelliferum*. However, more clones must be investigated because ploidy investigations of the haptophytes *C. polylepis* (Edvardsen and Vaulot 1996) and *E. huxleyi* (Green et al. 1996) have demonstrated that one morphotype in a suspected/demonstrated life cycle can represent more than one ploidy level. The differences in the banding pattern of the third intron within one calmodulin gene between the haploid strain of *Chrysochromulina polylepis* and all strains belonging to either *P. parvum* or *P. patelliferum* indicate that this may be so in our case. Thus, how the two *Prymnesium* species possibly are linked in a life cycle requires further investigation.

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