J. Phycol. **39,** 379–389 (2003)

PIGMENT SIGNATURES AND PHYLOGENETIC RELATIONSHIPS OF THE PAVLOVOPHYCEAE (HAPTOPHYTA)[1]

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Variations in the HPLC-derived pigment composition of cultured Pavlovophyceae (Cavalier-Smith) Green et Medlin were compared with phylogenetic relationships inferred from 18S rDNA sequencing, morphological characteristics, and current taxonomy. The four genera described for this haptophyte class (*Diacronema* Prauser emend. Green et Hibberd, *Exanthemachrysis* Lepailleur, *Pavlova* Butcher, and *Rebecca* Green) were represented by nine different species (one of which with data from GeneBank only). Chlorophylls *a*, c_1, c_2 and MgDVP (Mg-[3,8-divinyl]-phytoporphyrin-13^2-methylcarboxylate) and the carotenoids fucoxanthin, diadinoxanthin, diatoxanthin, and β,β-carotene were detected in all cultures. Species only differed in the content of an unknown (diadinoxanthin-like) xanthophyll and two polar chl *c* forms, identified as a monovinyl (chl c_1-like) and a divinyl (chl c_2-like) compound. This is the first observation of the monovinyl form in haptophytes. Based on distribution of these two chl *c* forms, species were separated into Pavlovophyceae pigment types A, B, and C. These pigment types crossed taxonomic boundaries at the generic level but were in complete accordance with species groupings based on molecular phylogenetic relationships and certain ultrastructural characteristics (position and nature of pyrenoid, stigma, and flagella). These results suggest that characterization of the pigment signature of unidentified culture strains of Pavlovophyceae can be used to predict their phylogenetic affinities and vice versa. Additional studies have been initiated to evaluate this possibility for the haptophyte class Prymnesiophyceae.

Key index words: **18S rDNA; chlorophyll *c* pigments; divinyl chlorophylls; Haptophyta; monovinyl chlorophylls; Pavlovophyceae; phylogeny**

[1]Received 7 May 2002. Accepted 4 December 2002.

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Knowledge of photosynthetic pigment systems is essential to our understanding of taxonomic and biological relationships within phytoplankton groups and can be used for the estimation of phytoplankton abundance in field populations using a chemotaxonomic approach based on multiple pigments (Mackey et al. 1996). Pigment analyses of natural field samples and unialgal cultures are now commonly performed using reverse-phase HPLC (High Performance Liquid Chromatography).

All oxygenic photosynthetic organisms synthesize a monovinyl (MV) or a divinyl (DV) form of chl *a*, and the sum of these two compounds (total chl *a*) is therefore commonly used as a proxy for total phytoplankton biomass. Each species synthesizes a specific suite of additional pigments (chl types and carotenoids), which have a light-harvesting or photoprotective function (Demers et al. 1991, Falkowski and Raven 1997). The composition of these accessory pigments is notoriously similar in all terrestrial plants but more complex and with a higher degree of variability among phytoplankton species. Despite this diversity, similarities generally increase toward the lower taxonomic levels (Jeffrey and Vesk 1997). This indicates that the complex metabolic processes required for pigment synthesis must be genetically coded. Pigments are hence not only practical chemotaxonomic markers for specific phytoplankton groups in aquatic ecosystems, but also of potential use in phylogenetic reconstructions.

Since the mid-1960s, cultured haptophytes have been the subject of many pigment studies (Jeffrey and Allen 1964), and applications of advances in the analytical field revealed an extraordinary diversity in associated pigments not observed for any other taxonomic group. Associated pigments include the carotenoids diadinoxanthin (Ddx), diatoxanthin (Dtx), β,β-carotene, β,ϵ-carotene, fucoxanthin (Fx) (Jeffrey and

Allen 1964, Norgård et al. 1974), 19′-hexanoyloxyfucoxanthin (HFx) (Arpin et al. 1976), 4-keto-Fx (Egeland et al. 1999), and 4-keto-HFx (Garrido and Zapata 1998, Egeland et al. 2000). The presence of the latter was previously mentioned by Arpin et al. (1976) but then suggested to be 19′-hexanoyloxy-paracentrone 3-acetate. Further pigments include chl c_1 and c_2 (based on elution order with chromatographic method used; Jeffrey 1969, 1972), a not yet identified chl *c*-like (Fawley 1989) and MV and DV forms of chl c_3 (Jeffrey and Wright 1987, Fookes and Jeffrey 1989, Garrido and Zapata 1993, 1998, Garrido et al. 1995). In addition to these polar (nonesterified) chl types, two different nonpolar chl *c* pigments were detected in *Emiliania huxleyi* (Nelson and Wakeham 1989) and *Chrysochromulina polylepsis* (Zapata et al. 1998), respectively. These compounds were recently identified as a chl c_2 moiety esterified to a monogalactosyldiacylglyceride bearing a different combination of two fatty acid residues (Garrido et al. 2000, Zapata et al. 2001). Based on combinations of accessory pigments, a range of haptophyte pigment types were defined (Jeffrey and Wright 1994, Garrido 1997, Rodríguez 2002).

Comparison of DNA sequences during the last years resulted in outstanding advances in understanding phylogenetic relationships among haptophyte species and members from other taxonomic groups. Combining molecular studies with detailed analyses of pigments should provide a robust approach to determine phylogenetic relationships in algal groups. Moreover, this approach can be used to evaluate evolutionary development of the photosystem.

In the present work, these possibilities were evaluated using cultured representatives of the haptophyte class Pavlovophyceae (Cavalier-Smith) Green et Medlin. This class has been largely ignored in phylogenetic studies but is a common component of coastal phytoplankton populations. This group was selected because it comprises a limited number of species with minor, but well-defined, morphological differences, currently classified in one order (Pavlovales Green), one family (Pavlovaceae Green), and four genera (*Diacronema* Prauser emend. Green et Hibberd, *Exanthemachrysis* Lepailleur, *Pavlova* Butcher, and *Rebecca* Green).

The class Pavlovophyceae was erected by Cavalier-Smith (1993) based on the order Pavlovales Green. This class name was validated by Edvardsen et al. (2000), who considered that the 6% divergence in the rRNA gene that separates the two major clades of the haptophyte algae is in agreement with that found among classes in other algal divisions. Within the haptophyte algae, the deep divergence of the two classes provides a model of key interest for evolutionary studies. In this context, the Pavlovophyceae are generally accepted to be representative of the primitive state, with characteristics most likely to be related to those of the ancestral haptophyte. Several distinct morphological features are common to all or most members of the Pavlovophyceae that separate them from members of the Prymnesiophyceae Hibberd emend. Cavalier-Smith. The most obvious of these is the markedly anisokont nature of the flagella and the relatively simple arrangement of microtubules and fibrous roots of the pavlovophyceaen flagellar-haptonematal basal complex (Green and Hori 1994). In addition, when scales occur in the Pavlovophyceae, they are not of the plate scale type found in the Prymnesiophyceae but rather consist of small dense bodies. These so-called knob-scales are considered to be modified scales (Green 1980) or modified hairs (Cavalier-Smith 1994), which often form a dense investment on the longer flagellum together with fine microtubular hairs. The mitotic process in the Pavlovophyceae also differs from that of the Prymnesiophyceae (Green and Hori 1988, Hori and Green 1994). Medlin et al. (1997), using a molecular clock calibrated from the coccolithophorid fossil record, placed an average time of divergence for the two classes of the Haptophyta at about 420 million years ago from an 18S rRNA phylogeny.

Biochemical support for the clear distinction of the two haptophyte classes has been provided by the detection of unusual dihydroxysterols (steroidal diols termed "pavlovols"), which are restricted to species from the Pavlovophyceae (Véron et al. 1996, Volkman et al. 1997). Considerable ecological diversity is apparent within the Pavlovophyceae, members of which are found in oceanic, coastal, brackish, and freshwater environments, and individual species may also be widely distributed geographically (Green 1980).

MATERIALS AND METHODS

Experimental strains and culture conditions. Monoclonal strains of the haptophyte species *Diacronema vlkianum* Prauser emend. Green et Hibberd (HAP 67), *Exanthemachrysis gayraliae* Lepailleur (HAP 15), *Pavlova gyrans* Butcher emend. Green et Manton (HAP 28), *Pavlova lutheri* (Droop) Green (HAP 44 = PLY75, UTEXLB1293), *Pavlova pinguis* Green emend. Green (HAP 19), *Pavlova virescens* Billard (HAP 16), and *Pavlova* sp. (HAP 33) were obtained from the Algobank culture collection (University of Caen, France). Of these, *E. gayraliae*, *P. virescens*, and *P. lutheri* are type strains. *Pavlova* sp. (HAP 33), an undescribed marine species, is provisionally named *P.* "*pseudogranifera*" because of similarities to the freshwater species *P. granifera* (Mack) Green (Billard, personal communication). All strains were grown in Tris II enriched seawater medium (Cosson 1987) at room temperature (20° C) under natural illumination (maximum 35 μmol photons·m^{-2}·s^{-1}) provided by a north-facing window. Taxonomic identity was confirmed by TEM, and culture purity was verified under high magnification in a light microscope.

Pigment analyses. Algal cells were harvested during the logarithmic phase of growth by vacuum filtration onto (25-mm GF/F) Whatman (Kent, UK) glass fiber filters and stored frozen (−80° C) until extraction. Filters were immersed in 3.0 mL 90% HPLC-grade acetone SdS (Peypin, France) and stored (24 h at −10° C) for passive pigment extraction. Final extraction procedures were performed with an ice-cooled cell homogenizer (5 min), followed by a centrifugation step (15 min at 5° C) to remove cell debris and filter fragments. Particle-free samples of 1.0 mL were transferred to 2.0-mL amber injection vials, stored at 5° C, and diluted by the addition of 0.2 mL water (Milli-Q water) (Waters S. A., Molsheim, France) just before HPLC sample injection. The dilution step was required to avoid peak distortion effects (Zapata and Garrido 1991), which at this degree of ace-

tone dilution did not occur at injection volumes up to 150 μL. Even though the higher dilution rates commonly used in the literature allow larger injection volumes (thus reducing detection limits), they inevitably induce moderate to severe pigment losses because of precipitation (Latasa et al. 2001). Pigment analyses were performed with a Thermo Separation Products chromatograph (currently Thermo Finnigan, San Jose, CA, USA), comprising a model P2000 solvent module, a UV3000 absorbance detector, an FL2000 fluorescence detector, an SN4000 controller, and a refrigerated (5° C) A/S-3000 autosampler. The method used was that described by Zapata et al. (2000b) but adapted to the HPLC setup used by modifications of the (binary) solvent gradient. This method was selected because it resolves all pigments currently described for the Haptophyta (including MV and DV forms of polar and nonpolar chl and the novel carotenoids 4-keto-HFx and 4-keto-Fx). Eluents (HPLC grade, SdS) used were 1) methanol-acetonitrile-aqueous pyridine solution (0.25 M pyridine, adjusted to pH 5.0 with acetic acid; 50:25:25, v/v/v) and 2) acetonitrile-methanol-acetone (60:20:20, v/v/v). The solvent gradient (linear steps, flow rate 1.0 $mL\cdot min^{-1}$) was programmed as follows (time, %): 0 min, 0%; 10–23 min, 23%; 25 min, 32%; 35 min, 40%; 38 min, 75%; 46 min, 85%; 48–55 min, 100%; 57 min, 0%. The stationary phase used was a Symmetry (Waters, Milford, USA) C_8 column (150 × 4.6 mm i.d., 3-μm particle size), protected with a guard column containing the same stationary phase, and maintained at a temperature of 25° C using a water jacket (Alltech, Deerfield, IL, USA) connected to a recirculating water bath (Julabo F12-MV, Julabo, Allentown, PA, USA).

For quantitative pigment analyses the UV3000 absorbance detector was programmed to collect data at a fixed wavelength (440 nm). Peak identities of each sample were evaluated during a second run using the scanning mode (400–750 nm, 1-nm resolution) of the detector (not being a diode-array model). Absorbance characteristics of the eluent, collected close to each peak, were subtracted from spectral data obtained at the corresponding peak maximum. Although such corrections improved quality of all spectra, they were mainly introduced for evaluation of minor compounds that could not be identified without such adjustments. These procedures required manual export procedures to a spreadsheet program, because they were not supported by the Chromquest v2.51 software (Thermoquest Corporation, Manchester, UK) software used (ChromQuest v. 2.51). Retention times and absorbance characteristics of peaks detected were compared with those of authentic standards (used for calibration procedures of the equipment), obtained from DHI (Hørsholm, Denmark), and co-chromatography of several algal species with well-known pigment compositions (Zapata et al. 2000b).

18S rDNA sequences and phylogenetic analyses. Genetic studies involved analyses of the above mentioned strains of *P. lutheri*, *P. virescens*, *P. "pseudogranifera,"* *D. vlkianum*, and *E. gayraliae* (GenBank nos. AJ515247, AJ515248, AJ515249, AJ515246, and AJ515250, respectively). Additional data from *Pavlova gyrans* Butcher emend. Green et Manton, *Rebecca salina* (N. Carter) Green (previously known as *Pavlova salina*; Edvardsen et al. 2000), and *Pavlova* sp. (CCMP 1416) were obtained from GenBank (nos. U40922, L34669, and AJ243369, respectively). The cultures were harvested during the logarithmic phase of growth by centrifugation (5 min at 3000 rpm). The pellet was resuspended in 1.0 mL of 100 mM Tris, pH 8.0, 100 mM NaCl, and 50 mM $Na_2EDTA\ 2H_2O$ and immediately frozen by immersion in liquid nitrogen. Samples were than stored at −80° C until processing. Genomic DNA was purified with "Dneasy Plant Minikit" (Qiagen, Hilden, Germany), following the instructions of the manufacturer, but with a modification of the lysis step during which a (1:1) mixture of 212–300 μm and 425–600 μm acid-washed and sterilized glass beads (Sigma-Aldrich, Munich, Germany) was added to the samples, followed by two grinding steps using a vortex (20 s each at 2500 rpm). DNA was eluted in 10 mM Tris-HCl (pH 8.0) from which 1–10 ng was used for PCR. Conditions for PCR reactions were as follows: 94° C, 5 min (94° C, 2 min; 50° C, 2 min; 74° C, 4 min; plus 2 extra seconds accumulating in every cycle) 30 times; 74° C, 10 min. These reactions were performed with an Amplitaq DNA Polymerase kit (Applied Biosystems, Foster City, CA, USA) and a Master Cycler Gradient (Eppendorf, Wesseling-Berzdorf, Germany), using sample volumes of 100 μL. The amplified DNAs were subsequently cleaned with a Qiaquick PCR Purification kit (Qiagen, Hilden, Germany). Sequences from the PCR templates (using both DNA strands) were obtained using an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA) and dye terminator cycle sequencing kits (Perkin-Elmer Co.; reactions run by Seqlab, Accelrys, Cambridge, UK).

Four oligonucleotides were used as sequencing primers of each template: those that were also used in the amplification reactions, 1F: 5′-aacctggttgatcctgccagt and 1528R: 5′-tgatccttctgcaggttcacctac from Medlin et al. (1988), plus two internal ones, F743: 5′-tgggataatgaaataggac and R783: 5′-cccctaactttcgttcttg. The quality of electropherograms were checked when editing sequences of DNA and revised for confirmation of newly observed substitutions. Xesee 3.2 software (Eric Cabot, Weizmann Institute of Science, Rehovot, Israel) was used to align the sequences manually during collection and edition, and electropherograms were viewed with the Chromas 1.45 program (Conor McCarty, Weizmann Institute of Science, Rehovot, Israel).

The eight Pavlovophyceae sequences were aligned with 52 other sequences obtained for members of the haptophyte class Prymnesiophyceae (Sáez et al., unpublished data) using ClustalX 1.81 software (Weizmann Institute of Science, Rehovot, Israel) (using default values, Thompson et al. 1997). The use of this large data set was intended to ensure that the quality of the alignment was optimal by using as many sequences as available. The sequences from the Pavlovophyceae were then realigned manually, for which the 18S rRNA secondary structure model (Medlin et al. 1988) was taken into account (i.e. nucleotides with the same structural position were considered to be homologous). Highly variable regions of the alignment were considered unreliable and thus excluded from phylogenetic analyses. This reduced the number of characters (nucleotide positions in the DNA alignment) from 1753 to 1657. *Emiliania huxleyi* (GenBank no. M87327) was selected as an outgroup, because this species is a member of the member of the haptophyte class Prymnesiophyceae, which is sister to the Pavlovophyceae.

Four main classes of analyses were performed: maximum parsimony (MP), maximum likelihood (ML), minimum evolution (ME), and Bayesian. MP procedures were carried out in four different ways: without any weighting, with weighting of characters only, with weighting of substitution types only (A/C, A/G, etc.), and with a combination of the last two. Weight assignations were performed with MacClade 3.04 software (Maddison and Maddison 1992) using default options. For this analysis a consensus tree was first derived (from an unweighted MP tree and a neighbor-joining tree produced with Kimura two-parameter distances; Kimura 1980). Each character was then weighted inversely to the minimum number of steps necessary to explain its phylogenetic distribution on the consensus tree. The substitution types were weighted inversely to their observed frequency on the same consensus tree. Modeltest 3.06 software (Posada and Crandall 1998) was used to select the model used for nucleotide substitution under ML. Such models of evolution quantify the probability that a particular mutation will take place, for example, increasing the chances of transitions versus transversions or allowing different characters to have different rates of mutations. Modeltest searches for an approximate evolution model using two independent statistical tests: the hierarchical likelihood ratio tests and the Akaike information criterion. The hierarchical likelihood ratio tests selected the Tamura-Nei model (Tamura and Nei 1993) with equal base frequencies, a strong heterogeneity in the mutation rate across sites (gamma shape = 0.53), and a 0.68 proportion of invariable sites. The Akaike information criterion test, on the other hand, selected the general time reversible model (Rodríguez et al. 1990), from hereafter referred to as GTRIG, with similar values for the gamma distribution parameter (0.52) and proportion of invari-

able sites (0.67). Kimura two-parameter and LogDet (Lockhart et al. 1994) distances and ML estimated distances (using settings derived from models selected by the Modeltest) were used for ME searches of the best tree. All analyses involved branch and bound searches, except when bootstrapping under ME and ML, for which heuristic searches were applied. All tree-building methods explained above were implemented with PAUP* 4.0b8 (Swofford 2000), using default settings unless otherwise stated.

At a later stage of our analyses, and to test our hypothetical tree of Figure 3, we ran a Bayesian search for the trees with "higher posterior probabilities" (Huelsenbeck and Ronquist 2001). We ran that search using the GTRIG with an undefined gamma distribution, during 500,000 generations, and saving every 100th tree. We discarded the first thousand trees, and the remaining 4000 trees, all with higher posterior probabilities, were used to construct a consensus tree. On this tree, "credibility values" for each clade are shown, which represent the percentage, of those 4000 trees, having the corresponding clades.

RESULTS

Pigment analyses. The chromatogram obtained for *P. gyrans* (Fig. 1A) revealed the presence of the characteristic polar chl *c* form (from hereafter referred to as chl c_{PAV}) first recorded in this species (Fawley 1989). Further compounds included MgDVP (a precursor in chl *a* and chls c_1/c_2 synthesis; Porra et al. 1997), chl c_1, chl c_2, Fx, Ddx, Dtx, and β,β-carotene. The pigment composition of *P. gyrans* was dominated by the carot-enoids Fx and Ddx (together near 30% of total pigment load; Table 1). Compared with the other species analyzed, *P. gyrans* contained relative large amounts of chlorophyllide *a* (chlide *a*), a phytol-free (and thus polar) degradation product of chl *a* (Jeffrey 1997), and three nonpolar chl *a* derivatives (allomers). These compounds were always detected independently of the growth phase during sample collection and were also observed in a methanol-extracted strain of this species previously analyzed by Zapata et al (2000b). These derivatives are thus considered common for this species rather than being indicative of the state of the culture and/or formation during erroneous extraction procedures. The *P. gyrans* strain used in this study contained an unidentified carotenoid (Unk-1; peak 10), with on-line absorbance characteristics (λ_{max} [411], [425], 446, 478 nm; values between brackets denote shoulders) identical to those collected for Ddx. Concentrations of Unk-1 were therefore estimated using calibration curve of Ddx. Unk-1 was also observed in the chromatogram of *P. pinguis* (Fig. 1B), which synthesized the same pigment as *P. gyrans*. Analysis of *P.* "*pseudogranifera*" (Fig. 1C) also revealed the same pigments detected in *P. gyrans*, but Unk-1 and β,β-carotene were in this case minor compounds only (Table 1). The pigments Unk-1 and chl c_{PAV} were not observed in the chromatograms of *P. lutheri* (Fig. 1D), *P. virescens* (Fig. 1E), and *D. vlkanium* (Fig. 1F), but the other pigments remained identical. The chromatogram obtained for *E. gayraliae* (Fig. 1G) revealed all pigments previously detected in *P. gyrans*, *P. pinguis*, and *P.* "*pseudogranifera*" but differed in that chl c_{PAV} was now separated in two individual peaks. Retention time and absorbance characteristics of the fast eluting compound (Fig. 2A) were identical to the chlxc_{PAV} form detected in *P. gyrans*, *P. pinguis*, and *P.* "*pseudogranifera*." The additional slow eluting compound was characterized by a different absorbance maximum of band III (Fig. 2B). Even though the maxima of the slow eluting form were only approximations (due to low concentrations), absorbance data were indicative for two different pigments, having similar molecular structures. Both chl c_{PAV} forms were characterized by a near equal intensity of bands I and II, which indicates that their molecular structures should closely resemble those of chls c_1 and c_2 rather than a chl c_3 form (in which band II is much more intense than band I). The main difference between the fast and slow eluting chl c_{PAV} form was that the band III absorbance maximum of the latter was notably shifted toward larger wavelengths (456 versus 452 nm). This shift suggested the presence of a DV/MV chl *c* pair, which was in agreement with the observed differences in retention times. With the HPLC protocol used, DV forms of all polar and nonpolar chl types eluted slightly before their MV analogues. Overall results thus revealed that the chl c_{PAV} form detected in *P. gyrans* is a DV (chl c_2-like) chl c_{PAV} form, whereas the additional peak detected in *E. gayraliae* is its MV (chl c_1-like) analogue. The molecular structures of these two pigments remain open for future studies, but following Porra et al. (1997) the enzymes required for their biosynthesis are possibly analogues of those used to produce chl *b*.

The relative contribution of pigments detected to total pigment load (including chl *a* derivatives) and concentrations normalized to chl *a* were calculated (Table 1) as a contribution to future field studies of phytoplankton populations using CHEMTAX software (Mackey et al. 1996). Species analyzed in the present work did not reveal a trace of HFx, 4-keto-HFx, 4-keto-Fx, MV, and DV forms of chl c_3 or nonpolar chl c_2 types. These compounds can thus be classified as typical pigments of the class Prymnesiophyceae. Observed variations in pigment composition were used to determine Pavlovophyceae pigment types, which were subsequently compared with phylogenetic results (Fig. 3) and certain morphological characteristics (Table 2) used for taxonomic identification.

DNA phylogenetic analyses. The determined phylogeny of the Pavlovophyceae based on ML analysis of 18S rDNA (GTRIG model) is shown in Figure 3. This phylogenetic "tree" (used as a synonym of "topology," because branch lengths were not considered) was supported by nearly all methods used. A few methods generated alternative positions for the species *D. vlkianum*, *R. salina*, and *E. gayraliae.* With unweighted and character-weighted MP, *D. vlkianum* was placed in the clade formed by *P. gyrans*, *P.* "*pseudogranifera*," and *Pavlova* sp. (CCMP 1416). However, this alternative was probably related to a bias among the nucleotide substitution classes (a typical problem of MP methods), because MP supported the position of *D. vlkianum* shown in Figure 3 when correcting for nucle-

TABLE 1. Concentrations of pigments (accessory compounds were normalized to chl *a*) and their relative contribution (%) to total pigment load (sum of all compounds).

Species	chl[1] *a*	DV-chl c_{PAV}	MV-chl c_{PAV}	MgDVP	chl c_2	chl c_1	Fx	Ddx	Dtx	Unk-1	β,β-carotene	Chlide	ΣAllom.
	(μg·L^{-1})	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)	(norm.)
Pavlova gyrans	61.0	0.03	x	0.0015	0.03	0.08	0.42	0.26	0.05	0.035	0.11	0.123	0.052
Pavlova pinguis	97.7	0.04	x	0.0004	0.03	0.12	0.62	0.32	0.02	0.109	0.24	0.017	0.046
Pavlova pseudo.	71.6	0.03	x	0.0010	0.02	0.07	0.43	0.22	0.02	0.001	0.01	0.018	0.021
Pavlova lutheri	192.8	x	x	0.0009	0.03	0.06	0.34	0.34	0.02	x	0.03	0.023	0.003
Pavlova virescens	32.6	x	x	0.0003	0.01	0.02	0.20	0.43	0.02	x	0.01	0.003	0.074
Diacronema vlkianum	64.0	x	x	0.0047	0.05	0.05	0.31	0.27	0.01	x	0.10	0.009	0.002
Exanthemachrysis gayraliae	104.9	0.01	0.005	0.0012	0.03	0.08	0.37	0.19	0.01	0.001	0.01	0.006	0.017
Mean	89.2	0.03	0.005	0.0014	0.03	0.07	0.39	0.29	0.02	0.037	0.07	0.028	0.031
							%						
Pavlova gyrans	43.6	1.5	x	0.06	1.2	3.5	18.4	11.3	2.1	1.53	4.7	5.4	2.3
Pavlova pinguis	37.8	1.7	x	0.01	1.1	4.5	23.4	11.9	0.8	4.11	8.9	0.6	1.7
Pavlova pseudo.	53.2	1.4	x	0.06	1.0	3.9	22.8	11.4	1.2	0.07	0.5	0.9	1.1
Pavlova lutheri	54.0	x	x	0.05	1.8	3.3	18.5	18.4	0.9	x	1.4	1.2	0.2
Pavlova virescens	52.4	x	x	0.02	0.4	0.8	10.3	22.7	1.0	x	0.5	0.1	3.9
Diacronema vlkianum	55.3	x	x	0.26	2.5	2.8	17.4	14.7	0.6	x	5.7	0.5	0.1
Exanthemachrysis gayraliae	56.3	0.6	0.3	0.07	1.6	4.5	21.0	10.9	0.8	0.04	0.8	0.3	1.0
Mean	50.4	1.3	0.3	0.08	1.4	3.3	18.8	14.5	1.0	1.44	3.2	1.3	1.5

Results indicated with "x" (pigment absent) were not included for calculating mean values.

otide substitution types. Substitution-weighted MP (and under character-plus substitution-weighted MP) placed the species *R. salina* and *E. gayraliae* together in one clade. This alternative was probably an artifact of parsimony known as long branch attraction (unrelated long branches "attract" to each other forming artefactual clades; Felsenstein 1978).

The external branches of *R. salina* and *E. gayraliae* were among the longest in our ingroup and therefore most susceptible to mutual attraction. We further attempted to test the tree of Figure 3 by running a recently developed tree-building method based on Bayesian inference (Huelsenbeck and Ronquist 2001). This method provided additional support for the tree topology in Figure 3. As shown by the "clade credibility values" in Figure 3 (fourth value provided along the branches of the tree), all its clades are clearly supported, although again the corresponding numbers are lower for the monophyletic group of *D. vlkianum*, *P. lutheri*, and *P. virescens* (52%). However, under our Bayesian analyses, the relative position of *R. salina* and *E. gayraliae* in Figure 3, a second less stable aspect of that tree, is strongly supported (89%). We conclude that our analysis clearly favors the tree topology depicted in Figure 3, because an overwhelming majority of our analyses support it and because there are some reasons to suspect why some of the tree-building methods used did not support two of the internal nodes (see above). Despite that, further data from other genes and/or other species would be useful to confirm our results.

DISCUSSION

Defining pigment types. Chls *a*, MgDVP, c_1, and c_2 and the carotenoids Fx, Ddx, Dtx, and β,β-carotene were common for all species of Pavlovophyceae analyzed in this study. This composition, with no additional compounds, was here classified as "*Pavlovophyceae pigment-type A*," which includes *P. lutheri*, *P. virescens*, and *D. vlkianum.* According to the four haptophyte pigment types defined by Jeffrey and Wright (1994) and the more detailed seven-type scheme defined by Garrido (1997), these species would be included in "*haptophyte type 1*." This is the simplest pigment composition detected for a member of this division and usually considered to be characteristic for diatoms rather than haptophytes. However, with the exception of chl c_1 (found in a limited number of haptophyte species only) Pavlovophyceae type A pigments comprise the basic pigment load of all haptophyte species (Garrido 1997, K. Van Lenning, unpublished data), including those classified in the class Prymnesiophyceae. Pavlovophyceae species with type A pigments plus Unk-1 and DV-chl c_{PAV} were here classified as "*Pavlovophyceae pigment-type B*." This group includes *P. gyrans*, *P. pinguis*, *P.* "*pseudogranifera*," and *R. salina* (Rodríguez 2002). With the exception of Unk-1 (not previously detected), this combination corresponds to that of "*haptophyte type 3*" defined by Garrido (1997). The simultaneous content of type B pigments plus MV-chl c_{PAV} (the most complex composition observed in the present work) was classified as "*Pavlovophyceae pigment-type C*" and includes only one species, *E. gayraliae.* This is the first observation of MV-chl c_{PAV} in a member of the Haptophyta and thus defines a new haptophyte pigment type.

The described Pavlovophyceae signatures A to C were based on pigment content only; relative proportions were not considered because they are known to vary according to irradiance conditions and nutrient concentrations (Stolte et al. 2000). MgDVP was detected (at least at trace levels) in all species but not considered to be a relevant parameter for determina-

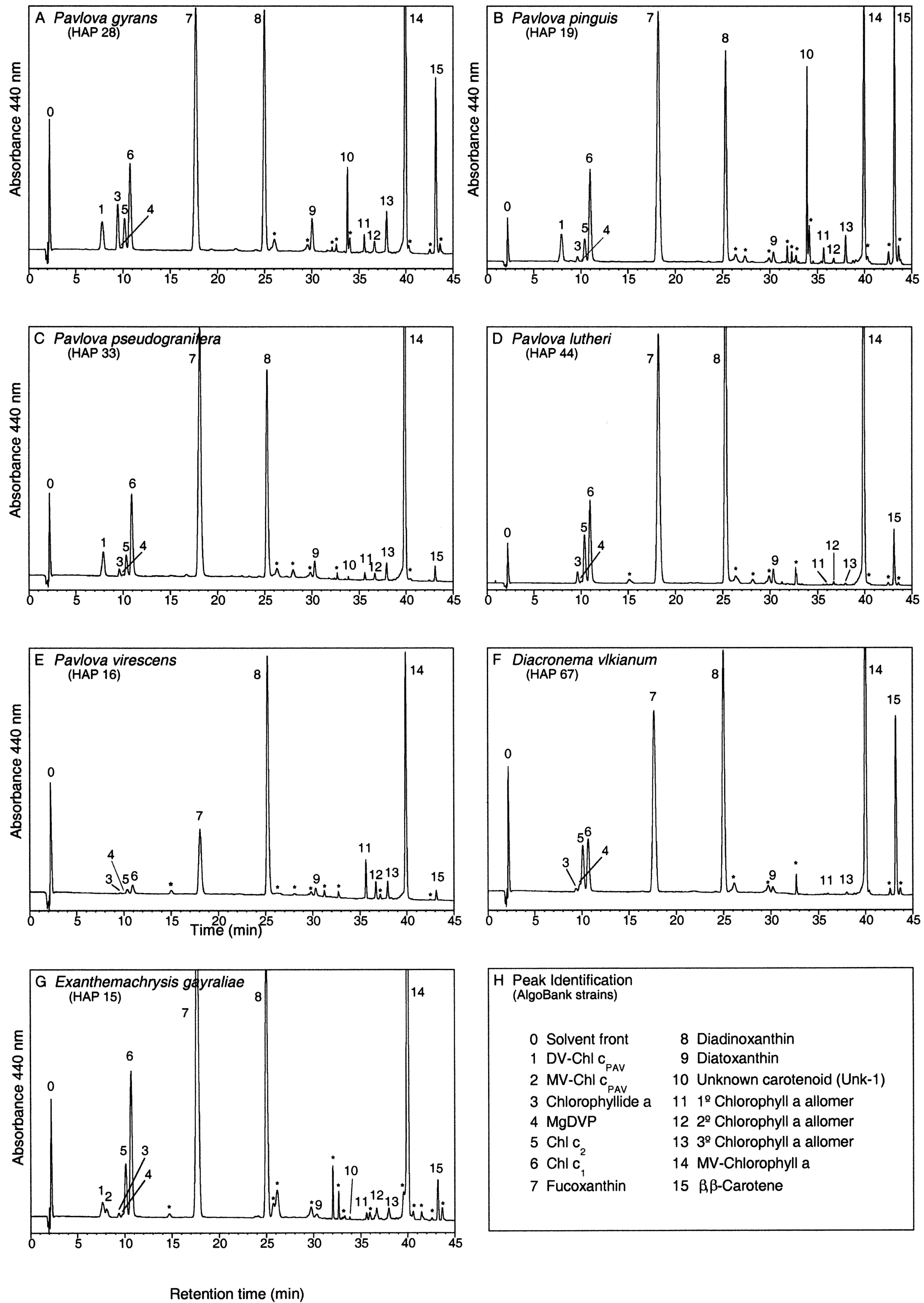

FIG. 1. HPLC chromatograms (A–G) of pigment extracts from seven different species (three genera) of the haptophyte class Pavlovophyceae and corresponding peak identifications (H). *Unidentified compounds.

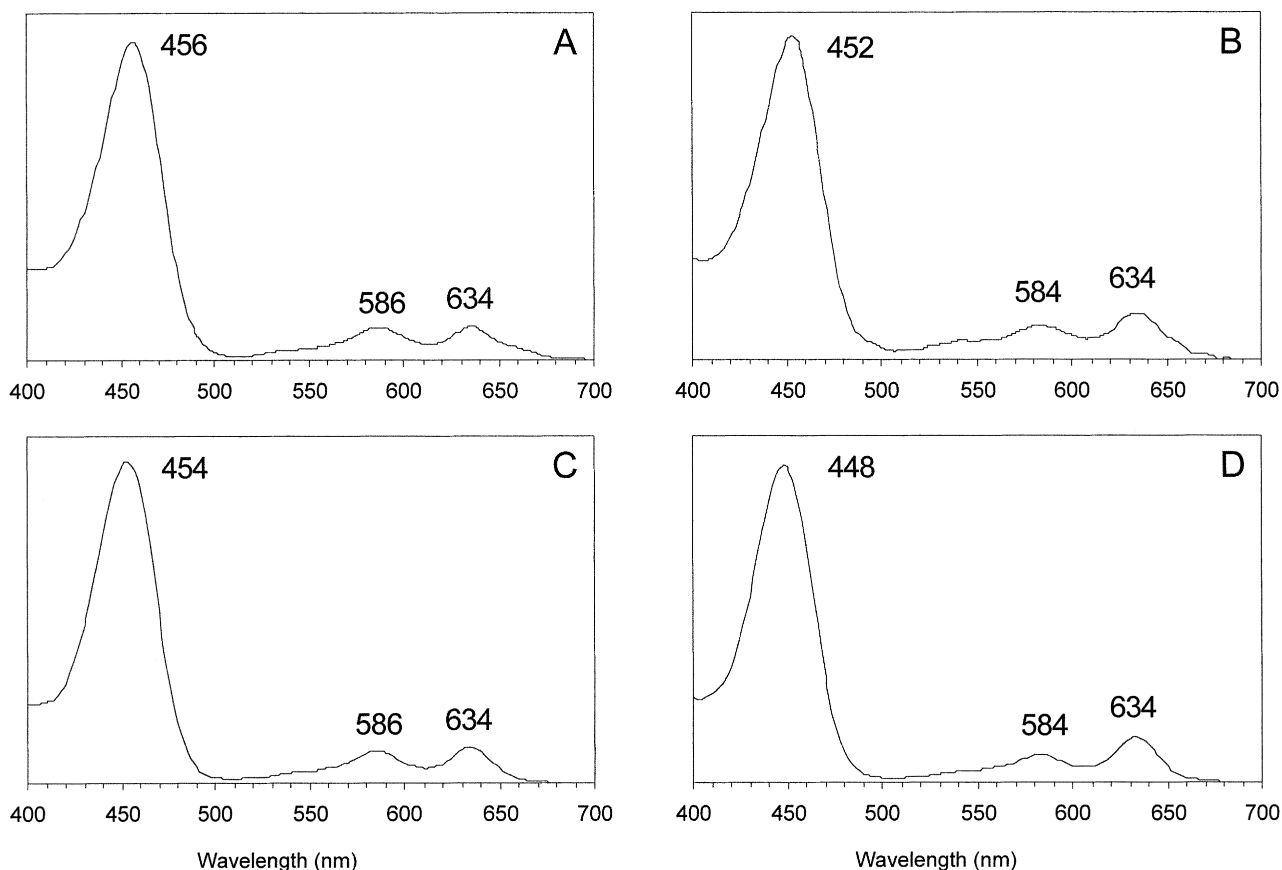

FIG. 2. Absorbance spectra (in eluent) of the polar chl *c* pigment detected in *Pavlova gyrans* (A), its counterpart detected in *Exanthemachrysis gayraliae* (B), together with the spectrally similar chl c_2 (C) and chl c_1 (D).

tion of pigment types. This compound is a precursor in chl *a* and chls c_1/c_2 synthesis (Porra et al. 1997), and hence its presence can be expected in extracts of any algal species.

Pigment types versus molecular phylogeny. Proposed species grouping based on molecular data showed three main clusters that were supported by differentiation-based pigment types A, B, and C. The congruency between 18S rDNA data and pigment types across the species studied suggested that evolutionary changes in pigment compositions among the Pavlovophyceae are rare. The described pigment types can thus be concluded not to result from temporal adaptations to specific ecological niches and can be used to obtain some degree of phylogenetic information (and vice versa). Using this approach the pigment content of *P. pinguis* should thus be indicative for a species related to type B rather than type A *Pavlova*. It was not possible to verify this hypothesis because our attempts to amplify 18S rDNA from *P. pinguis* failed for unknown reasons. The phylogenetic position of *Pavlova* sp. (CCMP1416) (revealed with results available in the GenBank) should be indicative for a Pavlovophycae pigment-type B species.

Evolution of pavlovophyceaen pigment types. Previous molecular analyses of cultured haptophytes performed by Edvardsen et al. (2000) and Fujiwara et al. (2001) indicated deep divergences within the Pavlovophyceae, although they only included two strains identified at the species level. Data obtained in the present work thus comprise the first results allowing a detailed evaluation of the evolutionary relationships within this algal class and their grouping based on pigment signatures (i.e. "chemotaxonomy"). Based on the comparisons of living species it is possible to evaluate evolution of pigment patterns. Assuming a parsimonious evolution (in which changes are rare) the number of steps (gain or losses of pigments) needed to explain the current content of living species should be minimal (Maddison and Maddison 1992). Based on this approach and the determined phylogenetic relationships, evolution toward the current Pavlovophyceae pigment types requires a minimum of two steps. Differentiation of pigment type A was subsequently attributed to loss of DV-chl c_{PAV} (and Unk-1) in an intermediate ancestor (one step), rather than assuming development of these compounds toward type B *Pavlova* spp., *R. salina*, and *E. gayraliae*

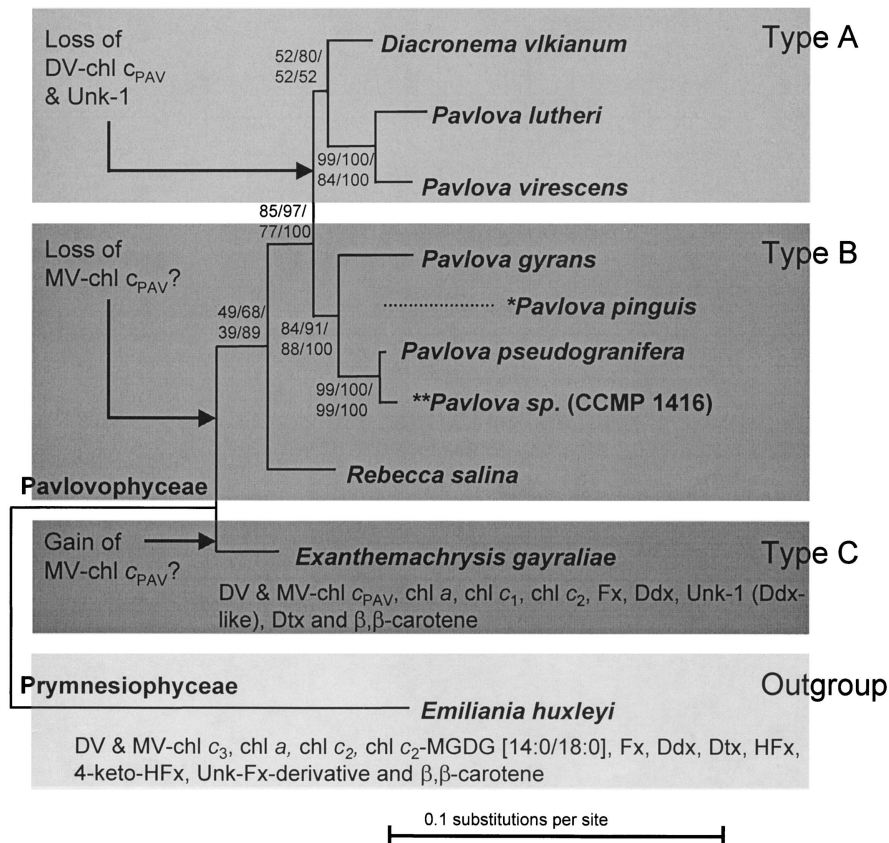

FIG. 3. Pavlovophyceae pigments types A to C superimposed on phylogeny based on ML analysis of 18S rDNA (GTRIG model). Complete pigment composition is indicated for outgroup and type C, whereas deviation (leading to type A) is indicated with an arrow. Bootstrap values (500 replicates) are shown for ML (with GTRIG), ME (1000 replicates; using distances estimated by ML and GTRIG), and weighted parsimony (1000 replicates; and weighting character and substitutions types). The fourth number provided along the branches of the tree shows the clade credibility values of Bayesian analysis. HFx, 19′-hexanoyloxyfucoxanthin; Fx, fucoxanthin; Ddx, diadinoxanthin; Dtx, diatoxanthin; Unk-1, unknown Ddx-like carotenoid. *Only submitted to pigment analysis; **only submitted to 18S rDNA analysis.

(three steps). The evolutionary origin of MV-chl c_{PAV}, on the other hand, is more difficult to establish, because both alternatives (development toward one type C species or loss in the line toward type B species) require one step only. However, because MV-chl c_{PAV} and its DV analogue are also synthesized by a few diatoms and dinoflagellates (Zapata et al. 2000a, Rodríguez 2002), the last option is more likely. A common evolutionary origin of such compounds in widely separated taxonomic groups is not unlikely, because some dinoflagellates possess plastids originating (from tertiary endosymbioses) from haptophytes (Tengs et al. 2000). However, the possibility of independent evolution of these compounds can of course not be excluded.

Pigment types versus morphology. The relatively simple pigment signature of the Pavlovophyceae, the presence of MV and DV-chl c_{PAV}, and the lack of many pigments commonly found in the Prymnesiophyceae provide further evidence for the class level separation within the haptophytes. The four genera within the class Pavlovophyceae currently contain just 12 described species (Jordan and Green 1994, Edvardsen et al. 2000). Within the class, however, there is considerable diversity in ecology, cell shape and size, presence or absence and shape of scales on the cell body and/or long flagellum, haptonemal length, and presence or absence and structure of the pyrenoid and the stigma (Green 1980). Despite this diversity, Green (1980) defined some broad groupings on fine structural grounds. The most obvious group contains *P. gyrans*, *P. pinguis*, and *P. granifera*, all with a bulging pyrenoid and a stigma on the internal face of the chloroplast. *Pavlova lutheri*, *P. virescens*, *P. calceolata* Veer, *P. noctivaga* (Kalina) Veer et Leewis and *D. vlkianum* form another group with stigmata, or at least stigma-like aggregations of osmiophilic bodies, on the outer face of the chloroplast but lack a pyrenoid. *Pavlova ennorea* Veer et Leewis and *R. helicata* (Veer) Green, on the other hand, appear to have no stigmata but have small protruding pyrenoids on the inner face of the chloroplast. *Rebecca salina* has neither stigma nor pyrenoid, and both this species and *R. helicata* have a very reduced (vestigial) posterior flagellum, the characteristic feature of the genus. *Exanthemachrysis gayraliae*, which has a bulging pyrenoid situated ventrally near the flagellar insertion and an unusual stigma-like structure consisting of a collection of osmiophilic vesicles lying at the junction between the pyrenoid stroma and the thylakoid-containing stroma of the chloroplast, is differentiated principally on the grounds that, unlike species of *Pavlova*, the motile cells lack flagellar knob scales and the nonmotile cells lack external flagellar apparatus (Gayral and Fresnel 1979).

A broad correlation exists between these morphological groupings and pigment types A to C defined in

TABLE 2. Comparison of pigment types defined in this study with morphological characteristics of the Pavlovophyceae.

Pigment type (this study)	Species	Pyrenoid (position relative to chloroplast, nature)	Stigma (nature, position relative to chloroplast)	Additional features
Type A	***D. vlkianum***	Absent	Inconspicuous, outer face	Swelling on posterior flagellum
	P. lutheri	Absent	Inconspicuous, outer face	None
	P. viriscens	Absent	Inconspicuous, outer face	None
	? P. calceolata	Absent	Inconspicuous, outer face	None
	? P. noctivaga	Absent	Inconspicuous, outer face	None
Type B	***P. gyrans***	Posterior, bulging	Conspicuous, inner surface	None
	P. pinguis	Posterior, bulging	Conspicuous, inner surface	None
	P. "pseudogranifera"	Posterior, bulging	Conspicuous, inner surface	None
	? P. granifera	Absent	Conspicuous, inner surface	None
	R. salina	Inner surface, protruding	Conspicuous, inner surface	Vestigial posterior flagellum
	? R. helicata		Absent	
Type C	***E. gayraliae***	Ventral bulging	Osmiophilic vesicles, transition between chloroplast and thylakoid stroma	Nonmotile cells lack flagellar apparatus
?	*P. ennorea*	Inner surface, protruding	Absent	None

Species in bold were analyzed in this study. For pigment types A and B, ? represents predicted pigment type groupings.

this study (Table 2). Pigment profiles support the morphological grouping of *P. gyrans*, *P. granifera* (as *P.* "*pseudogranifera*"), and *P. pinguis* but are not sufficient to distinguish this group from *R. salina* (all four being type B). The distinct separation of *R. salina* in our molecular genetic phylogeny is, however, in accordance with the removal of this species from *Pavlova* and the establishment of the genus *Rebecca* by Edvardsen et al. (2000). *Rebecca helicata* and *P. granifera* are predicted to possess type B pigments, and *Pavlova* sp. (CCMP1416), which should be *P. pinguis*, *P. granifera*, or an undescribed species, is likely to have a posterior bulging pyrenoid and a conspicuous stigma located on the inner surface of the pyrenoid. The pigment type A species included in the present study belong to the second group defined by Green (1980). The type A genus *Diacronema*, containing only *D. vlkianum*, is distinguished by the unique structure of the posterior flagellum, which consists of a proximal part swollen on the side adjacent to the cell (Green and Hibberd 1977). In both the pigment signature based and the genetically defined groupings, *D. vlkianum* falls in the same clade as *P. lutheri* and *P. virescens*. These species also have certain morphological features in common (lack of pyrenoid, stigma-like structure on outer face of chloroplast; see Table 2), but *Diacronema* also retains certain unique features, such as the structure of the short flagellum, that separates it from the other species in the clade. The placement of *D. vlkianum* basal to these *Pavlova* species receives only moderate bootstrap support, and thus the taxonomic significance of the flagellar structure in the former is emphasized. *Pavlova calceolata* and *P. noctivaga* are predicted to contain type A pigments; pigment and genetic analyses of these two species would be useful for the resolution of the taxonomy of this clade relative to other *Pavlova* species. Four possibilities can be envisaged: 1) *D. vlkianum* could be transferred to *Pavlova* if certain morphological (presence/absence and structure of pyrenoid and stigma) and pigment (presence/absence of DV-chl c_{PAV}) features are not considered taxonomically relevant; 2) *P. lutheri* and *P virescens* (and potentially *P. calceolata* and *P. noctivaga*) may be transferred to an emended genus *Diacronema* if the latter morphological and pigment groupings are considered relevant and the *D. vlkianum* flagellar morphology does not prove to be significant; 3) a new genus may be created to contain *P. lutheri* and *P virescens* (and potentially *P. calceolata* and *P. noctivaga*) if all these features are considered relevant (work in progress by John Green); and 4) the current taxonomy can be retained, leaving *Pavlova* as a paraphyletic, but nonpolyphyletic, genus. Of these, option 3 is the most desirable phylogenetically and morphologically. Pigment type C contains only *E. gayraliae*, a species that is also clearly distinct from other members of the Pavlovophyceae in the genetic phylogeny. The generic separation based on morphological characters (see above) is therefore warranted, and the presence of MV-chl c_{PAV} does appear to have fine-scale taxonomic significance. Gayral and Fresnel (1979) transferred the one species not thus far considered, *P. ennorea*, together with *P. noctivaga* to the genus *Exanthemachrysis* on the basis that motile cells lacked flagellar knob scales and nonmotile cells apparently lacked external flagellar apparatus. Green (1980) adopted a more conservative approach of retaining these species in *Pavlova* because the flagellar apparatus of *P. ennorea* was only described as "incomplete" with little detail given by Van Der Veer and Leewis (1977), whereas Kalina (1975) described and illustrated flagella and haptonema as present in both

motile and nonmotile cells of *P. noctivaga*. As mentioned above, the latter species is predicted on ultrastructural grounds to group with pigment type A species. Analysis of the pigment signatures of these two species, specifically determination of the presence or absence of DV and MV-chl c_{PAV}, would help resolve their taxonomic affinities.

Conclusions. Pigment analysis of nine species of the haptophyte class Pavlovophyceae resulted in discrimination of three pigment types in this low diversity (based on described number of species) but ancient algal group. The phylogenetic tree resulting from parallel molecular genetic analysis was completely congruent with species classification based on pigment types and morphological characteristics. Parsimonious analysis of pigment evolution based on molecular results suggested that differences in determined Pavlovophyceae pigment types resulted form gradual simplification of the light-harvesting antenna during evolution. Current taxonomic classification of the Pavlovophyceae was broadly supported by phylogeny and pigment types, but the genus *Pavlova* appeared paraphyletic, and taxonomic revision is warranted. Combining molecular genetics with a chemotaxonomic and a morphological approach appeared to be a highly productive methodology for studying the evolutionary diversity of algae.

We thank Prof. Chantal Billard for providing information on the undescribed species *P.* "*pseudogranifera*" and for useful comments on the manuscript, Bánk Beszteri for discussions on phylogenetic methods, and Jarone Pinhassi, Manuel Zapata, and two anonymous reviewers for suggestions and final corrections. This research was funded by the EU via the CODENET (Coccolithophorid Evolutionary Biodiversity and Ecology Network) project of the FP4 TMR activity.

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