New haptophyte lineages and multiple independent colonizations of freshwater ecosystems

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Summary
The diversity and ecological relevance of small haptophytes in marine systems is increasingly recognized. Similar investigations in freshwater remain scarce, despite some recent studies showing the existence of divergent haptophyte lineages and indicating that these microalgae can occur at high abundance in lakes. We studied the diversity of haptophytes in a wide variety of marine, salty continental and, most particularly, freshwater environments by amplifying, cloning and sequencing 18S rRNA genes. For this purpose, we designed two sets of primers specific for the two recognized haptophyte classes, Prymnesiophyceae and Pavlovophyceae. We detected pavlovophyte sequences only in freshwater systems as well as several novel prymnesiophyte phylotypes in both freshwater and marine environments. In addition, we retrieved a cluster of sequences (HAP-3) from the Marmara Sea branching deeply in the haptophyte tree with no clear affiliation to either of the two recognized classes. Five of the freshwater prymnesiophyte phylotypes detected formed a divergent monophyletic group (EV) without close described representatives that branched within the Isochrysidales, a group of generally marine and most often calcifying coccolithophorids. The presence of several sequences of freshwater haptophytes scattered among marine taxa in phylogenetic trees confirms the occurrence of several independent haptophyte transitions between marine and freshwater environments.

Introduction
Haptophytes are unicellular aquatic, mostly marine, photosynthetic eukaryotes. They are broadly distributed and recognized as key players in biogeochemical cycles in marine ecosystems. Their wide distribution and relative high abundance in marine waters was initially shown by high-performance liquid chromatography analyses of its characteristic pigment 19'-hexanoyloxyfucoxanthin (19-Hex) (Andersen et al., 1996). According to recent molecular investigations, haptophytes contributed from 30% to 50% of the photosynthetic standing stocks in the photic layer across the oceans in the year 2000 (Liu et al., 2009). Similarly, targeted metagenomic analyses suggest that haptophytes constitute on average 25% of global eukaryotic picophytoplankton carbon biomass (Cuvelier et al., 2010). Haptophytes may play a significant role in primary production even when they are not dominant; for instance, a new group of haptophytes was recently shown to contribute significantly to CO₂ fixation despite its low relative abundance (Jardillier et al., 2010). This might be due to their higher growth rates and their bigger size as compared with other more abundant planktonic phototrophs (Cuvelier et al., 2010) and, perhaps, also to the presence of efficient carbon-concentrating mechanisms (Reinfelder, 2011). Haptophytes may also play additional roles in the C-cycle through heterotrophic pathways, as some haptophytes have been shown to be mixotrophic (Legrand et al., 2001; Frias-López et al., 2009).

In addition to their increasingly recognized ecological importance in oceans, haptophytes are at the heart of a phylogenetic debate, as their position in the eukaryotic tree remains unresolved. Haptophytes were initially affiliated to the chromalveolates, a eukaryotic super-group including also alveolates, stramenopiles (heterokonts) and cryptophytes, the ancestor of which was thought to have acquired a red algal plastid as secondary endosymbiont (for review, see Keeling, 2009). However, whereas the monophyly of alveolates and stramenopiles is easily retrieved, recent molecular phylogenetic analyses suggest that cryptophytes and haptophytes do not form a monophyletic group with them. They rather seem to be sister to other clades, such as the kathablepharids, telonemids and centrohelid heliozoa, which do not have known photosynthetic members, forming another super-group recently named Hacrobia (Okamoto et al., 2009) or CCTH group (cryptophytes, centroheliozoa, telonemids, haptophytes) (Burki et al., 2009), even if its monophyly remains discussed (Burki et al., 2012). At a finer phylogenetic scale, several questions also remain open. Haptophytes currently encompass two classes, the...
Pavlovophyceae and Prymnesiophyceae. The monophyly of those classes is supported by both morphology and molecular data, although the phylogenetic relationships within each class are often reshuffled (see Edvardsen and Medlin, 2007; Bendif et al., 2011; Edvardsen et al., 2011 for recent propositions). Pavlovophytes have mainly been observed in littoral and brackish environments, although they have also been detected in freshwater systems by amplification of their 18S rRNA genes using pavlovophyte-specific primers (Shalchian-Tabrizi et al., 2011). They are currently represented by only four genera (namely Diaicronema, Exanthemachrysis, Pavlova and Rebecca) distributed in four clades according to phylogenies based on 18S rRNA genes (Bendif et al., 2011). Compared with pavlovophytes, prymnesiophytes display a higher diversity and abundance and, consequently, have been studied more thoroughly. They comprise the orders Coccolithales, Isochrysidales, Phaeocystales, Prymnesiales, Syracosphaerales and Crepidolithales (De Vargas et al., 2007) or Zygodiscales (Jordan et al., 2004; Edvardsen and Medlin, 2007) depending on the authors. The legitimacy of the orders Syracosphaerales and Crepidolithales or Zygodiscales is under discussion; they might be included within the Coccolithales (Edvardsen and Medlin, 2007). Several haptophyte genera based on cell morphology later revealed to be polyphyletic based on molecular analyses (Edvardsen et al., 2011) and, conversely, several morphological species were found indistinguishable based on molecular markers (Bendif et al., 2011), which urges for a revision of haptophyte systematics based on reliable molecular phylogenies.

In spite of their ecological and phylogenetic importance, the diversity of haptophytes is not fully explored. Since the beginning of the century, the use of molecular methods based on the amplification, cloning and sequencing of 18S rRNA genes has uncovered a vast diversity of marine protist lineages affiliating to known taxa and a few undescribed divergent lineages scattered in the eukaryotic tree (López-García et al., 2001; Moon-van der Staay et al., 2001; Massana and Pedros-Alio, 2008). However, in those studies using general eukaryotic primers, the diversity and novelty of haptophytes was rather low. In the particular case of haptophytes, Moon-van der Staay and colleagues (2000) showed a great discrepancy between the proportion of haptophytes in marine pico-plankton based on their characteristic 19-Hex pigment and their relative representation in 18S rDNA libraries. It is well known that the use of lineage-specific primers may enhance the recovery of diverse environmental sequences within a given taxon, as has been shown, for example, for cercozoans (Bass and Cavalier-Smith, 2004) or diplonemids (Lara et al., 2009). Actually, the use of specific primers targeting the 28S rRNA gene allowed the discovery of an unsuspected diversity of non-calcifying prymnesiophytes in oceanic waters (Liu et al., 2009). The enrichment of haptophyte fractions by flow cytometry has been another alternative showing a hitherto unveiled diversity within the group. In this way, a novel clade of highly divergent haptophytes was discovered in South Pacific waters (Shi et al., 2009). Subsequent metagenomic studies on haptophyte fractions sorted by flow cytometry confirmed a wide prymnesiophytes diversity in oceans (Cuvelier et al., 2010).

Compared with marine ecosystems, haptophytes in freshwater systems have been less studied and seem less diverse. Only a dozen freshwater haptophyte species have been described, whereas over 400 species have been defined from marine environments (Preisig, 2002). Most research efforts have concentrated on toxic species, as they trigger massive fish kills (Hansen et al., 1994; Edvardsen and Imai, 2006). Described freshwater haptophytes belong to the orders Pavlovalales, Coccolithales and Prymnesiales (Preisig, 2002), with Chrysochromulina and Prymnesium being the most common genera. Consistent with classical observations, molecular diversity studies of small eukaryotes in lakes (Richards et al., 2005; Lepère et al., 2008; Triadó-Margarit and Casamayor, 2012) revealed neither a broad diversity nor a high proportion of haptophyte sequences, most of the sequences retrieved being close to Chrysochromulina parva. Nonetheless, under certain conditions, lake haptophytes can reach relatively high proportions in the euhtotic zone. For instance, haptophytes accounted for 7.7% of microbial eukaryotes in Lake Aydat and up to 62.8% of small planktonic protists in Lake Bourget (Lepère et al., 2010). Most studies on freshwater haptophytes have concentrated in lakes. However, freshwater environments are varied and highly heterogeneous, and the eukaryotic diversity in these ecosystems is far from being described, especially in small freshwater bodies. In one of the first protist molecular surveys carried out in freshwater systems, Šlapeta and colleagues (2005) studied two ponds with different redox status and retrieved 18S rDNA phylotypes that formed a monophyletic group with pavlovophytes and prymnesiophytes but branched deeply in the haptophyte clade. The occurrence of this group (HAP-1) in freshwater systems has been confirmed by subsequent studies in Scandinavian lakes using 454 pyrosequencing of the V4 region of 18S rRNA genes (Shalchian-Tabrizi et al., 2011). This same study also suggested, based on the premise of a marine origin for haptophytes, that several transitions between marine and freshwater systems have occurred along the evolutionary history of the group. However, as the diversity and distribution of haptophytes in different ecosystems across a salinity gradient is not well known, this hypothesis awaits confirmation.

To contribute to a comprehensive account of haptophyte diversity, we designed new specific 18S rDNA primers...
primers covering the diversity of known prymnesiophytes, pavlovophytes and the rest of known haptophyte environmental lineages. We then carried out molecular surveys in a variety of ecosystems across a salinity gradient, including oceanic samples, a variety of freshwater lakes and ponds, as well as brackish and hypersaline shallow lakes. Our study confirms a wider diversity of haptophytes in marine, as compared with freshwater, ecosystems, and reveals the occurrence of novel divergent lineages in both marine and freshwater systems. The ecological distribution of the new haptophyte lineages further supports the hypothesis of multiple transitions from marine to freshwater systems.

Results and discussion

Prymnesiophyte and pavlovophyte diversity and distribution

In order to study haptophyte diversity in a variety of ecosystems, we first designed specific 18S rDNA primers targeting separately Prymnesiophyceae and Pavlovo-
phyceae plus other divergent lineages (see supplementary Materials and methods). Specific haptophyte primers have been used in previous studies. However, they targeted either the 28S rRNA gene (Liu et al., 2009), for which a comprehensive species and environmental refer-
sampling is missing, or a particular haptophyte subset (pavlovophytes) (Shalchian-Tabrizi et al., 2011). Using our more inclusive haptophyte primer sets, we then amplified 18S rRNA genes from a variety of samples including freshwater, marine and saline systems (Table 1) (see Supporting information for methodological details). In all continental samples, haptophyte 18S rDNAs were amplified only after nested PCR reactions, suggesting that haptophytes were in low abundance in these ecosystems. Although we cannot totally discard a PCR bias, this suggests that haptophytes were in low abundance in these ecosystems. However, we cannot totally discard a PCR bias, this is in agreement with the paucity of haptophyte sequences (see Supporting information for methodological details). In all continental samples, haptophyte 18S rDNAs were amplified only after nested PCR reactions, suggesting that haptophytes were in low abundance in these ecosystems. Although we cannot totally discard a PCR bias, this is in agreement with the paucity of haptophyte sequences (see Supporting information for methodological details).

More than half the 32 OTUs detected in the environments sampled were not closely related to described species (Table S2). Among them, five marine OTUs were affiliated to previously described clades without any cultured representative species (Prym_14, Prym_20) or even to potential new clades (Prym_13 and Prym_29, Ma135_Pav3) (Figs 2 and 3, Table S2).

All sequences forming OTUs Prym_13 and Prym_29 were detected in the Sea of Marmara. They were grouped with the environmental sequence F01N5 that was retrieved in our study were affiliated to clade 4 (Bendif et al., 2011) (Fig. 3). Although the pavlovophyte diversity and distribution observed in our study were reduced, these results are consistent with current knowledge (Bendif et al., 2011).

New insights in the diversity of marine haptophytes

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Operational taxonomic units Prym_14 and Prym_20 were affiliated to prymnesiophyte clade D and clade E respectively (Edvardsen et al., 2000; 2011), which are exclusively composed of marine environmental sequences from the South East Pacific Ocean and the Equatorial Pacific Ocean (Edvardsen et al., 2000; Moon-van der Staay et al., 2000; Shi et al., 2009). Over the last decade, the phylogenetic position of those two clades D and E changed with the increasing number of sequences retrieved in marine environments (Edvardsen et al., 2000; Moon-van der Staay et al., 2000; 2001). Recently, Shi and colleagues (2009) proposed clade D to form an independent lineage at the base of all other prymnesiophyte orders within the haptophytes. In addition to its widespread
distribution in marine environments, our study confirms that these sequences form an independent clade among Prymnesiophytes, and should therefore represent a new order (Fig. 2). Shi and colleagues (2009) also proposed clade E to be a sister group of the coccolithophorid orders (Coccolithales, Isochrysidales, Syracosphaerales). Our phylogenetic analysis confirms the placement of the clade within the clade formed by coccolithophorid orders, but without any clear sisterhood to any of the described orders (Fig. 2). The inclusion of new coccolithophorid sequences should help place clade E within one of the described orders or support the erection of a novel order for organisms of this clade.

The use of pavlovophyte-specific primers revealed a divergent lineage, OTU Ma135_Pav3, grouping 60 clone sequences (Table S2), only detected at surface (15 m depth) in the Sea of Marmara. The position of this divergent OTU is unstable. It branched either as a sister group of pavlovophytes, although with very low support (Fig. S2), or at the base of both pavlovophytes and
prymnesiophytes (Fig. 3). The closest 18S rDNA sequence retrieved by a BLAST search against the Silva database (Pruesse et al., 2007) was retrieved from the South East Pacific Ocean (Shi et al., 2009) and shared only 93.9% identity with it (Table S2). Nonetheless, environmental sequences FS14K073 and EN360CTD001 (Cuvelier et al., 2010) and SHAX 513 (Orsi et al., 2012), which only partially overlapped our sequences, shared 97–98% identity with our sequence Ma135-Pav3-C1 and clustered together in phylogenetic trees (Fig. S2). This divergent OTU Ma135_Pav3 did not branch with any of the other deeply divergent haptophyte lineages detected so far: the freshwater clade HAP-1 formed by the environmental sequences CV1-B1-97 and CV1-B2-32 retrieved from a suboxic pond (Šlapeta et al., 2005) and APB2H and AI9LL from Lake Finsevatn in Norway (Shalchian-Tabrizi et al., 2011) (Figs 3 and S2) and the marine clade HAP-2 including sequences from the Bioscope cruise T65.100 and T58.080 (Shi et al., 2009). Therefore, the newly detected clade, which we name here HAP-3, together with HAP-1 and HAP-2, might represent new class-level groups of haptophytes along with pavlovophytes and prymnesiophytes.

**Novel haptophytes in freshwater systems**

We detected a group of five OTUs unrelated to any described species or to any environmental sequence (Group EV, Fig. 2) in two freshwater lakes, the shallow lake Etang des Vallées and Lake Annecy (Table 1). Interestingly, this phylogenetic group affiliates to Isochrysidales, a coccolithophorid order that so far was thought to be composed exclusively of marine haptophytes. This order of haptophytes is composed of non-calcifying (*Isochrysis*) and calcifying (*Chrysotila, Emiliania, Gephyrocapsa*) genera. To our knowledge, *Hymenomonas roseola* (Coccolithales) is the only calcifying freshwater haptophyte that has been described (Manton and Peterfi, 2009).
The lack of 18S rRNA gene sequences of *H. roseola* prevents us from establishing the phylogenetic relationship between this species and the sequences detected in our freshwater systems. Nonetheless, there is little chance that the EV group, affiliated to Isochrysidales, turns out to be closely related to *H. roseola*, which has been classified within the Coccolithales based on classical morphological description. Future morphological identification of members of the group EV should show whether the former are actually calcifying or not.

Even if our phylogenetic analysis strongly supports its monophyly, the group EV is highly diverse. Indeed, sequences forming this group share 95% of identity only (average value calculated on 16 complete sequences).

For comparison, 18S rRNA sequences of the polyphyletic genus *Chrysochromulina*, which is scattered in the order Prymnesiales (Fig. 2), share 96% identity [average value calculated on full-length 18S rDNA genes from eight *Chrysochromulina* species by Caron et al. (2009)]. Sequences belonging to group EV were found in both 0.22–5 µm and 5–30 µm fractions at the Etang des Vallées (Table 1), with OTUs Prym_1 and Prym_3 being shared by both the size fractions, although more diversity was retrieved in the biggest cell-size fractions. There are three possible explanations for the observation of members of this group in different fractions. One explanation would be imperfect size fractionation that could happen if, for instance, the cells are fragile and lyse during

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**Fig. 2.** Maximum likelihood phylogenetic tree of 18S rDNA haptophyte sequences of marine, freshwater and salty continental habitats showing prymnesiophyte clades. A total of 758 non-ambiguously aligned positions were used to reconstruct the tree; gaps were excluded. Two cryptophyte sequences were used as outgroup. 18S rRNA gene sequences from this work are shown in bold. Pavlovophyte and HAP-1 branches are shown collapsed. Bootstrap values greater than 50% are shown at nodes. The scale bar represents the estimated number of substitutions per 100 positions per a unit branch length.

**Fig. 3.** Maximum likelihood phylogenetic tree of 18S rDNA haptophyte sequences of marine, freshwater and salty continental habitats showing the diversity of pavlovophytes and basal haptophyte lineages. A total of 851 non-ambiguously aligned positions were used to reconstruct the tree; gaps were excluded. 18S rRNA gene sequences from this work are shown in bold. The prymnesiophyte branch is showed collapsed. Bootstrap values greater than 50% are shown at nodes. The scale bar represents the estimated number of substitutions per 100 positions per unit branch length.
the filtration process, or can be deformed and pass through filters under the applied filtration pressure. Second, this could be explained by a diversity of cell sizes within members of group EV due, for instance, to the presence of smaller gametes or different cell sizes in different life cycle phases (e.g. haploid–diploid transitions). Finally, a third possibility would be the non-obligate physical association of those organisms with bigger ones (e.g. symbiosis). The fact that OTUs Prym_1, Prym_2, Prym_3 and Prym_4 form a tight and diverse subgroup with much longer branches than most other prymnesiophyte sequences in our phylogenetic tree (Fig. 2) might suggest that they are mutualistic or parasitic haptophytes, as symbiotic lineages tend to accelerate their evolutionary rate (Wernegreen, 2002; Bromham, 2009). Symbiotic prymnesiophytes have already been observed, for instance in association with the planktonic foraminifer Globigerinella siphonifera (Gast et al., 2000), which can either live with its host or be free-living.

This new group EV has been detected in two geographically distant and ecologically different freshwater ecosystems: the shallow and oligo-mesotrophic shallow lake Etang des Vallées and the deep and oligotrophic Lake Annecy (Fig. 1). Similarly, the divergent freshwater clade HAP-1 has also been detected in two different and geographically distant environments, first in the sediment of a suboxic pond in France (Šlapeta et al., 2005), then in the sediment of a high oligo- to mesotrophic alpine lake in Norway (Shalchian-Tabrizi et al., 2011). This suggests that, although not necessarily abundant, very divergent haptophyte lineages may still be found in the understudied freshwater systems. Investigating these lineages might help to reconstruct the evolutionary history of the group and to understand its ecology.

**Multiple independent marine–freshwater transitions**

The two pavlovophyte OTUs detected in this work were composed of sequences retrieved only from freshwater systems and close to the emended genus Diacroneema (clade 4) (Bendif et al., 2011). OTU Pav_1 was detected in three ponds (Fig. 1, Table S2) and had as closest BLAST hit the sequence of Corcontochrysis noctivaga [synonym of Diacroneema noctivaga (Bendif et al., 2011)] strain AC88 (Table S2), isolated from freshwater. It also seems close to sequences Finsevatn 89.12, Svaersvann14 and Svaersvann16, which were retrieved from freshwater (Shalchian-Tabrizi et al., 2011). OTU Pav_2 was only encountered in one lake (Fig. 1, Table S2) and affiliated to Diacroneema vikanium, which has been visually recorded in freshwater, brackish and marine habitats (Preisig, 2002). Among prymnesiophytes, OTUs Prym_5 and Prym_12 (Prymnesiales, clade B2) were only recorded in freshwater environments. OTU Prym_5 was affiliated to C. parva, a well-known and widely distributed freshwater toxic species (Hansen et al., 1994; Nicholls, 2003; Edvardsen and Imai, 2006; Luo et al., 2011). We detected C. parva sequences in seven out of the 15 freshwater ecosystems studied, thus confirming its broad distribution (Fig. 1, Table S2). Within the clade B2 of Prymnesiales, the OTU Prym_12, which was detected in the oligotrophic lake Annecy, clustered with environmental marine sequences (Fig. 2). The remaining freshwater OTUs Prym_1, Prym_2, Prym_3, Prym_4 and Prym_19 formed the group EV within the Isochrysidales, a prymnesiophyte order with no known freshwater representative. These freshwater haptophyte lineages may then represent five distinct transitions from marine to freshwater environments.

In addition to OTUs clustering within known haptophyte orders, the divergent lineage HAP-1 (Šlapeta et al., 2005; Shalchian-Tabrizi et al., 2011), together with the species Pavlova granifera (Green, 1973) and six OTUs affiliated to the clade B1 of Prymnesiales (Finsevatn AI7UI, Finsevatn AKXPZ, Finsevatn AYOY0H) or to the pavlovophyte clade 4 (Finsevatn 8912, Svaersvann 14 and 16) (Shalchian-Tabrizi et al., 2011), have only been found in freshwater systems and might represent other examples of putative marine–freshwater transitions. Altogether, there might have been at least nine freshwater colonization events from marine waters. In addition, a few species, for which the 18S rRNA gene sequences are not yet available, have been visually observed in freshwater systems such as Chrysochromulina Laurentiana, Chrysochromulina inornamenta, Chrysochromulina breviturrita (Hansen et al., 1994; Nicholls, 2003) and H. roseola (Manton and Peterfi, 1969).

Continental salty ecosystems also harbour particular haptophytes as shown by OTUs Prym_16 and Prym_24 (Fig. 2) that were isolated in a brackish pond (France) and from Chiprana, belonging to the hypersaline lake complex in the central Ebro basin (Spain) (Jonkers et al., 2003). OTU Prym_16 was affiliated to Jomonlithus littoralis ALGO Je5, a coastal marine species, while OTU Prym_24 was related to Pseudoisochrysis paradoxo CCAP949/1, isolated from the brackish York River Estuary in Virginia (USA) and Chrysothila lamellosa, a species often isolated from coastal marine regions (such as the strain ALGO HAP17) or brackish continental environments (such as CCAP 918/1 isolated in London, UK). Haptophytes recorded in salty continental systems thus appear to be phylogenetically close to species of either coastal habitats or continental brackish systems. This finding is in agreement with the importance of salinity as a barrier for marine–continental environment transitions, (Lozupone and Knight, 2007; Logares et al., 2009), in spite of the fact that haptophytes seem to have crossed that barrier several times in the course of their evolution.
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References


82 Prymnesiophyte sequences used to construct the tree are shown collapsed. The alignment contained 1678 selected positions (positions with less than 50% gaps were selected using BMGE on a 2131 bp alignment). 18S rRNA gene sequences from this work are shown in bold. Full circles indicate freshwater sequences; other sequences are from marine ecosystems. Bootstrap values greater than 50% are shown at nodes (1000 replicates). The scale bar represents the number of substitutions per 100 positions per unit branch length.

Table S1. Major characteristics of the samples analysed in this study. The positive or negative amplification of 18S rRNA genes with Prymnesiophyte (Prym.) or Pavlovophyte (Pav.)-specific primers is indicated with ‘+’ or ‘−’ signs. n.a., not applicable; n.d., not done.

Table S2. OTUs identified in this work. The name of representative sequences for each OTU, their first BLAST hit in the Silva Database SSU104, their percentage of similarity as well as the total number of sequences retrieved in each system are given.