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NEW MARINE MEMBERS OF THE GENUS *HEMISELMIS* (CRYPTOMONADALES, CRYPTOPHYCEAE)[1]

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Cryptomonads are a ubiquitous and diverse assemblage of aquatic flagellates. The relatively obscure genus *Hemiselmis* includes some of the smallest of these cells. This genus contained only two species until 1967, when Butcher described seven new marine species mainly on the basis of observations with the light microscope. However, from these seven taxa, only *H. amylifera* and *H. oculata* were validly published. Additionally, the features Butcher used to distinguish species have since been questioned, and the taxonomy within *Hemiselmis* has remained clouded due to the difficulty in unambiguously applying his classification and validating many of his species. As a result, marine strains are often placed into one of three species—*H. rufescens* Parke, *H. virescens* Droop, or the invalid *H. brunnescens* Butcher—based on cell color alone. Here we applied microscopic and molecular tools to 13 publicly available *Hemiselmis* strains in an effort to clarify species boundaries. SEM failed to provide sufficient morphological variation to distinguish species of *Hemiselmis*, and results from LM did not correlate with clades found using both molecular phylogenetic and nucleomorph genome karyotype analysis, indicating a high degree of morphological plasticity within species. On the basis of molecular characters and collection geography we recognize four new marine species of *Hemiselmis*—*H. cryptochromatica* sp. nov., *H. andersenii* sp. nov., *H. pacifica* sp. nov., and *H. tepida* sp. nov.—from the waters around North America.

Key index words: **cryptomonad; *H. andersenii* sp. nov.; *H. cryptochromatica* sp. nov.; *H. pacifica* sp. nov.; *H. tepida* sp. nov.; *Hemiselmis*; nucleomorph; phylogeny**

Abbreviations: **CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA; CrPC, cryptomonad phycocyanin; CrPE, cryptomonad phycoerythrin; PCC, Plymouth Culture Collection, UK**

Cryptomonads are a group of flagellate unicellular algae that inhabit a wide variety of freshwater, brackish, and marine habitats. Although somewhat variable in size, structure, trophic status, and pigmentation, cryptomonad cells are generally characterized by the presence of chl $a + c$–containing plastids (chloroplasts), a distinctive anterior furrow or gullet, and extrusive organelles called ejectisomes (Kugrens et al. 2000). The cryptomonads are of considerable interest to researchers investigating the origin and evolution of eukaryotic photosynthesis, as it is widely recognized that their plastids were acquired through the process of secondary endosymbiosis (i.e., the uptake and permanent retention of a eukaryotic endosymbiont; Delwiche 1999, Keeling 2004, Archibald and Keeling 2005, Archibald 2007). Four membranes surround the cryptomonad plastid with the space between the inner and outer membrane pairs—the periplastid space—corresponding to the remnant cytosol of the engulfed algal cell. Within the periplastid space, the remnant nucleus of the algal endosymbiont persists in a highly derived form, termed the nucleomorph. Cryptomonad nucleomorphs have been the subject of numerous studies aimed at better understanding their evolutionary origin and functional significance, most notably the complete sequencing of the nucleomorph genome of the model cryptomonad *Guillardia theta* D. R. A. Hill and Wetherbee. The *G. theta* nucleomorph genome is comprised of three chromosomes and is a mere 551 kilobase pairs (kb) in size, with little in the way of noncoding DNA and duplicated loci (Douglas et al. 2001). The presence of three nucleomorph chromosomes in cryptomonads appears to be a universal feature (reviewed by Archibald 2007).

The Cryptomonadales presently comprises ~20 genera containing more than 100 species. The genus *Hemiselmis* was first established by Parke (1949) when she described *H. rufescens* nearly 60 years ago, making *Hemiselmis* one of the more recently described genera of the group. A second species, *H. virescens*, was described by Droop (1955), adding a blue-green member to the genus. What started out as a small and relatively obscure genus was significantly expanded by Butcher (1967) in his monograph on the small algae of British coastal waters. In his manuscript, Butcher described seven additional marine species of *Hemiselmis* but provided multiple localities for many of his types, rendering all but *H. amylifera* and *H. oculata* invalid. He then

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separated *Hemiselmis* into two subgenera, solely on the basis of cell color, despite previous literature suggesting this character was of dubious taxonomic value (Pringsheim 1944).

In addition to the four valid marine species of *Hemiselmis* (*H. amylifera*, *H. oculata*, *H. rufescens*, and *H. virescens*), three freshwater taxa have been attributed to the genus. Butcher (1967) transferred two European species with tortured taxonomic histories (Silva 1980)—*Sennia parvula* Skuja (1948) and *Nephroselmis olivacea* sensu Pasher (1912) non F. Stein—into *Hemiselmis*, and the blue-green freshwater *H. amylosa* was described more recently (Clay and Kugrens 1999) from a Colorado lake. Despite the considerable diversity in pigmentation and habitat, relative to other cryptomonad genera, species of *Hemiselmis* display a characteristic kidney-shaped morphology, laterally inserted flagella, and genus-specific phycobiliprotein pigments, suggesting that members of the genus share a recent common ancestor.

Even at the level of ultrastructure, *Hemiselmis* species are difficult to diagnose. In an electron microscopic investigation, Santore (1982) questioned the interpretation of many of the morphological characters used by Butcher to define species. In particular, Santore's examination of the authentic strain of Butcher's *H. brunnescens* and comparison to the two earlier species, *H. virescens* Droop and the authentic strain of *H. rufescens* Parke, suggested that LM was inadequate for effectively delineating species among these diminutive cryptomonads. Unfortunately, a vast majority of *Hemiselmis* authentic cultures have been lost, and most of Butcher's species have never been tested, nor have their names been legitimized.

Four members of *Hemiselmis* have been included in comprehensive molecular phylogenies of the cryptomonads (Clay and Kugrens 1999, Deane et al. 2002), based on the SSU rDNA, and two species were used in subsequent phylogenies based on nucleomorph-encoded SSU rDNA (Hoef-Emden et al. 2002, Lane et al. 2006). Molecular phylogenies consistently group *Hemiselmis* with members of the genus *Chroomonas*, a nonmonophyletic assemblage of blue-green cryptomonads (Marin et al. 1998, Clay and Kugrens 1999, Deane et al. 2002, Hoef-Emden et al. 2002, Lane et al. 2006). These phylogenies suggest that *Hemiselmis* is composed of closely related species and is the only genus that has been shown, using molecular data, to contain both red and blue-green members. Despite the evolutionary implications of this observation—that species within a genus can change their cell color and pigment composition over relatively short timescales—there has been little follow-up work on *Hemiselmis* pigmentation or attempts to investigate this genus at a molecular level.

We have studied the molecular and karyotypic diversity of the nucleomorph genome of diverse cryptomonad species and recently demonstrated that *Hemiselmis rufescens* CCMP644 has an atypical nucleomorph genome structure (Lane and Archibald 2006). All investigated nucleomorph genomes of cryptomonads, as well as those of an unrelated algal lineage, the chlorarachniophytes, had previously been shown to possess subterminal rDNA cistrons on all six chromosome ends (Rensing et al. 1994, Zauner et al. 2000, Douglas et al. 2001, Gilson et al. 2006, Lane et al. 2006). In contrast, full rDNA cistrons are absent from the ends of chromosome II in three different species of *Hemiselmis* (Lane and Archibald 2006). Instead, only the 5S gene of the rDNA persists on the *Hemiselmis* chromosome II, presumably the result of a recent interchromosomal recombination event. This karyotypic feature has the potential to be a useful diagnostic tool for the systematics and taxonomy of *Hemiselmis*.

All of the previously studied marine species of *Hemiselmis* have been described from the waters around Great Britain. In this study, our goal was to assess the diversity of marine *Hemiselmis*, particularly strains from outside of northern Europe. Using LM, SEM, nucleomorph SSU rDNA sequence, and nucleomorph genome karyotype data, we recognized four new species of *Hemiselmis*—*H. cryptochromatica* sp. nov., *H. andersenii* sp. nov., *H. pacifica* sp. nov., and *H. tepida* sp. nov.—all from the coastal waters of North America and the Atlantic Gulf Stream. These data also indicate that Butcher's *H. brunnescens* is a morphological variant of *H. rufescens* and cast doubt on the morphological characters used to describe the seven marine species of *Hemiselmis* in his monograph (Butcher 1967).

MATERIALS AND METHODS

Cell cultures, DNA and phycobiliprotein extractions, and PCR. All available strains designated as belonging to the genus *Hemiselmis* (Table S1 in the supplementary material) were acquired from the following culture collections: Culture Collection of Algae and Protozoa (CCAP), Dunbeg, Argyll, UK; Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), Boothbay, Maine, USA; Plymouth Culture Collection (PCC), Plymouth, UK; and Roscoff Culture Collection (RCC), Roscoff, France. However, CCMP442 and 440 did not grow well enough to be included here. We also acquired two unidentified cryptomonad cultures that contained cells small enough ($<9 \times 5$ μm) to belong to *Hemiselmis*, unidentified cryptomonad cultures CCMP706 and 1181. Cultures were maintained in the medium recommended by their provider at both room temperature and 20°C with a 16:8 light:dark (L:D).

Phycobiliproteins were extracted by freezing pelleted cells from 50 mL of dense culture and then resuspending the cells in 0.2 M phosphate buffer (pH 6.8) once they were thawed. Freezing lysed the cells and released the phycobiliproteins into the buffer. Cellular debris was removed by centrifugation at 15,000*g* for 20 min, and absorption spectra were then recorded using a SpectraMax Plus 384 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA).

For PCR, cells were harvested by centrifugation from 50 mL of dense culture, and DNA was extracted using the procedures described in Lane et al. (2006). An ~1.7 kb fragment of the nucleomorph-encoded SSU rDNA gene was amplified, cloned,

and sequenced as described therein. Isotype DNA samples from new species described here have been submitted to the New York Botanical Garden (NYBG). DNA from all strains can also be obtained from the authors upon request.

Phylogenetic analysis. Newly obtained nucleomorph SSU rDNA genes were added manually to a preexisting alignment of diverse cryptomonad sequences (Lane et al. 2006). Phylogenetic analyses were performed in identical fashion on two alignments. The first alignment included 1,437 nucleotide positions from members of the genera *Hemiselmis* and *Chroomonas*, as well as a select group of outgroup taxa, including *Guillardia theta*, *Teleaulax amphioxia*, *Proteomonas sulcata*, and species of *Cryptomonas* (Table S1). *Chroomonas* is consistently associated with *Hemiselmis* in molecular analyses, and the other taxa have been variously resolved as sister to the *Chroomonas/Hemiselmis* clade (Marin et al. 1998, Deane et al. 2002, Hoef-Emden et al. 2002, Lane et al. 2006). To better resolve the intraspecific relationships within *Hemiselmis*, a second alignment was created including 1,675 positions from only members of this genus to take advantage of more variable data excluded from the first analysis. Each alignment was analyzed using maximum-likelihood (ML) and Bayesian algorithms, performed with PAUP v 4.0b10 (Swofford 2002) and MrBayes v 3.0 (Huelsenbeck and Ronquist 2001), respectively. Two hundred random sequence addition replicates were performed using the heuristic search option, tree-bisection-reconnection (TBR) branch swapping and the best fit model (TVM + I + Γ) as determined by Modeltest v 3.7 (Posada and Crandall 1998), although the resulting trees were identical in topology when a general-time-reversible (GTR) + I + Γ model was used. One thousand bootstrap replicates were performed with PhyML v. 2.4.4 (Guindon and Gascuel 2003) using the GTR model, four substitution rate categories, and invariable sites with all model parameters estimated during the analysis. MrBayes was run three times, independently for each alignment, for 1,000,000 generations using the GTR + I + Γ model. Trees were sampled every 100 generations, and the likelihood values stabilized at ~2,000 trees, which were discarded as ''burn-in.'' The remaining 8,000 trees were used to calculate the consensus tree.

Nucleomorph karyotype analysis. Pulsed-field gel electrophoresis (PFGE) was carried out as described previously (Lane et al. 2006). Briefly, 2 L of log-phase cell cultures (density = $1–2 \times 10^8$ cells$\cdot$L^{-1}) for each strain were harvested by centrifugation, embedded in low-melt agarose, and digested using the buffers described by Eschbach et al. (1991). Southern hybridizations were conducted using probes and methodology outlined in Lane and Archibald (2006).

LM and SEM. For differential interference contrast (DIC) and phase contrast LM, live cells were placed on glass slides and sealed with coverslips. Images were taken at ×100 magnification using a Zeiss (Toronto, Ontario, Canada) Axiovert 200 M microscope and an Axiocam HRc color digital camera. Subcellular features were interpreted based on comparisons to previous ultrastructural studies of *Hemiselmis* (Santore 1982, Clay and Kugrens 1999). For SEM, cells were gently pelleted (800*g* for 2 min) and resuspended at a 1:1 ratio in 5.0% gluteraldehyde, for a final concentration of 2.5%. Cells were fixed for 30 min, gently pelleted, and washed three times with filtered seawater before being resuspended in 2.0% OsO_4 and left for 30 min. The fixed cells were washed three times with freshwater and added to a 10 mL syringe. The water was pushed through a 0.25 M Millipore (Billerica, MA, USA) filter so that the cells remained on the filter. Cells were dehydrated in an ethanol series of 25, 35, 50, 70, 80, and 90% for 5 min each, followed by three 5 min washes of 100% ethanol. Filters were then cut into sections in 100% ethanol, critical-point-dried, and attached to stubs using carbon tape. The samples were coated with a gold/palladium mix with a Quarum Technologies (Newhaven, East Sussex, UK) SC7620 Mini Sputter Coater before being viewed with a Hitachi (Tokyo, Japan) S-4700, cold field emission scanning electron microscope.

RESULTS

Based on nucleomorph 18S rDNA sequencing and karyotype analysis of strains included in this study (Table 1), as well as morphological comparisons to previously described *Hemiselmis* species, it is evident that many of these cultures represent undescribed species. However, because of the variability in the appearance of subcellular features (observed with the light microscope) within clades reported here, the diagnoses must, for the sake of future comparisons, rely mainly on characters other than those previously used to separate species in this genus.

Hemiselmis andersenii C. E. Lane et J. M. Archibald, sp. nov.

Cellulae reniformes aspectu laterali et obovoideae aspectu dorsali, 5.5–8.5 μm altitudine ab 3–5 μm diametro extensae; pigmento Cr-phycoerythro 555; plastus parietalis, ab aurantiaco ad atrorubrum extensus; cellulae ex genomate nucleomorphi circa 572 partes kilobasium in magnitudine constantes; genoma nucleomorphi in tres chromosomomatibus dispositum, unum majorius multo quam duo chromosoma minora; exempla 18S rDNA tantum in extremis duobus chromosomomatibus maximis nucleomorphis et in extremo uno chromosomomatis minimi praesentia; 5S rDNA in extremis omnis sex chromosomomatibus nucleomorphis praesentia.

Cells reniform in lateral view and obovoid in dorsal view, ranging in size from 5.5 to 8.5 μm (length) by 3–5 μm (width); pigment Cr-phycoerythrin 555; plastid parietal, ranging from orange to dark red in color; cell containing a nucleomorph genome ~572 kb in size; nucleomorph genome encoded on three chromosomes, one of which is significantly larger than the two similarly sized, smaller chromosomes; copies of the 18S rDNA present only on both ends of the largest nucleomorph chromosome and one end of the smallest one; 5S rDNA present on all six nucleomorph chromosome ends.

Holotype: NYBG# SEM7.

Isotype material: SEM8; NYBG# 002357 (DNA).

Culture from which the type was observed: CCMP644.

Type location: 23.0000 N, 75.0000 W; Gulf Stream.

Other included strains: CCMP439, CCMP441, and CCMP1180.

Etymology: Named after Dr. Robert Andersen for his contributions to microalgal systematics and continued stewardship of the CCMP, without which this and many other studies of phytoplankton would not be possible.

Hemiselmis cryptochromatica C. E. Lane et J. M. Archibald, sp. nov.

Cellulae reniformes aspectu laterali et pyriformes ad obovoideis aspectu dorsali, 4.5–6.5 μm

TABLE 1. Taxonomic changes made to the *Hemiselmis* strains used in this study.

Previous strain name (culture designation)	Revised species designation	Collection location	Estimated nucleomorph genome size (kb)	Temperature maximum (°C)
Unidentified cryptomonad (CCMP1181)	*Hemiselmis cryptochromatica* C. E. Lane et J. M. Archibald sp. nov.	Boothbay Harbor, Maine, USA	572	<21
Hemiselmis virescens Droop (PCC157)	*Hemiselmis virescens*	Wend of Buckwater, Plymouth Sound, England	589	<21
Unidentified cryptomonad (CCMP706)	*Hemiselmis pacifica* C. E. Lane et J. M. Archibald sp. nov.	Friday Harbor, Washington, USA	595	<21
Hemiselmis brunescens Butcher (PCC14)	*Hemiselmis rufescens* Parke	Plymouth Sound, England	595	<21
Hemiselmis rufescens Parke (PCC563)	*Hemiselmis rufescens*	Off Port Erin, Isle of Man, British Isles	595	<21
Hemiselmis sp. (PCC631)	*Hemiselmis rufescens*	ST. L4, 50°15′ N, 4°13′ W, English Channel	595	<21
Hemiselmis sp. (RCC659)	*Hemiselmis rufescens*	Oslofjord, Norway	595	<21
Hemiselmis rufescens (CCMP644)	*Hemiselmis andersenii* C. E. Lane et J. M. Archibald sp. nov.	23.0000 N, 75.0000 W, Gulf Stream (approx.)	570	>25
Hemiselmis rufescens (CCMP439)	*Hemiselmis andersenii*	29.8000 N, 85.6666 W, (approx.) Cape San Blas, USA	570	>25
Hemiselmis sp. (CCMP441)	*Hemiselmis andersenii*	23.0000 N, 75.0000 W, Gulf Stream (approx.)	570	>25
Hemiselmis virescens (CCMP1180)	*Hemiselmis andersenii*	21.0000 N, 93.0000 W, (approx.) Mexico	570	>25
Hemiselmis virescens (CCMP442)	*Hemiselmis tepida* C. E. Lane et J. M. Archibald sp. nov.	29.3833 N, 94.8833 W, Galveston Channel, Texas, USA	560	>25
Hemiselmis virescens (CCMP443)	*Hemiselmis tepida*	29.3833 N, 94.8833 W, Galveston Channel, Texas, USA	ND	>25

Each strain is listed with its previous and revised species name. Additionally, information on the collection location, nucleomorph genome size, and culture temperature maximum are included.

altitudine ab 3.0–4.5 μm diametro extensae; pigmento Cr-phycocyano 630; in vitro, cellulae sine colore vel canae dilultae; genoma nucleomorphi circa 585 partes kilobasium in magnitudine, in tres chromosomomatibus aliquantum aequaliter separatis dispositum; 18S rDNA tantum in chromosomomatibus maximis et minimis nucleomorphis praesentia; 5S rDNA tantum in chromosomomatibus minimis et mediocribus nucleomorphis remanentia.

Cells reniform in lateral view and pyriform to obovoid in dorsal view, ranging in size from 4.5 to 6.5 μm (length) by 3.0 to 4.5 μm (width); pigment Cr-phycocyanin 630; in culture, cells lacking color to faint gray; nucleomorph genome ~585 kb in size, encoded on three somewhat similarly sized chromosomes; 18S rDNA present on only the largest and smallest of the three nucleomorph chromosomes; 5S rDNA persists only on the smallest and midsized chromosomes of the nucleomorph.

Holotype: NYBG# SEM3.

Isotype material: SEM4; NYBG# 002359 (DNA).

Culture from which the type was observed: CCMP1181.

Type location: 43.8441 N, 69.6413 W; Bigelow Laboratory dock, West Boothbay Harbor, Maine, USA.

Etymology: Named for an unusual lack of coloration, even in dense culture.

Hemiselmis pacifica C. E. Lane et J. M. Archibald, sp. nov.

Cellulae reniformes aspectu laterali et ovatae aspectu dorsali, 7.0–8.5 μm altitudine ab 4–6 μm diametro extensae; pigmento Cr-phycocyano 615; plastus parietalis, smaragdinus ad glaucescenti in colore extensus; genoma nucleomorphi circa 600 partes kilobasium in magnitudine, in tres chromosomatibus dispositum, unum majorius quam duo chromosoma minora; 18S rDNA tantum ab chromosomatibus mediocribus nucleomorphis absentia; 5S rDNA in chromosomomatibus omnis tres nucleomorphis praesentia.

Cells reniform in lateral view and ovate in dorsal view, ranging in size from 7.0 to 8.5 μm (length) by 4–6 μm (width); pigment Cr-phycocyanin 615; plastid parietal, grass green to gray-green in color; nucleomorph genome ~600 kb in size, encoded on three chromosomes, arranged as two large and one small; 18S rDNA absent only from the middle chromosome; 5S rDNA present on all three nucleomorph chromosomes.

Holotype: NYBG# SEM5.

Isotype material: SEM6; NYBG# 002356 (DNA).

Culture from which the type was observed: CCMP706.

Type location: 48.5440 N, 123.0100 W; Friday Harbor, San Juan Island, Washington, USA.

Etymology: This is the first species of *Hemiselmis* to be described from the Pacific Ocean.

Hemiselmis tepida C. E. Lane et J. M. Archibald, sp. nov.

Cellulae reniformes aspectu laterali et ovatae aspectu dorsali, 5.5–7.0 μm altitudine ab 3.5–4.5 μm diametro extensae; pigmento Cr-phycocyano 615; plastus parietalis, aeruginosus ad smaragdino in colore extensus; genoma nucleomorphi circa 560 partes kilobasium in magnitudine, exempla 18S rDNA tantum in chromosomomatibus maximis et minimis nucleomorphis praesentia; 5S rDNA in chromosomomatibus omnis tres nucleomorphis praesentia.

Cells reniform in lateral view and ovate in dorsal view, ranging in size from 5.5 to 7.0 μm (length) by 3.5–4.5 μm (width); pigment Cr-phycocyanin 615; plastid parietal, absinthe green to emerald green in color; nucleomorph genome ~560 kb in size, encoded on three chromosomes, arranged as one large and two small chromosomes; copies of the 18S rDNA present only on the largest and smallest nucleomorph chromosomes; 5S rDNA present on all three nucleomorph chromosomes.

Holotype: NYBG# SEM1.

Isotype material: SEM2; NYBG# 002358 (DNA).

Culture from which the type was observed: CCMP443.

Type location: 29.3833 N, 94.8833 W; Galveston Channel, Texas, USA.

Other included strains: CCMP442.

Etymology: Named for its warm-water collection site.

Molecular data. In an attempt to shed light on systematic relationships within *Hemiselmis*, we sequenced nucleomorph 18S rDNA from seven previously unstudied strains assigned to this genus, as well as two previously unnamed CCMP strains (1181 and 706). Phylogenetic analyses of these sequences in the context of sequences obtained from GenBank were consistent with previously published molecular phylogenies (Marin et al. 1998, Deane et al. 2002, Hoef-Emden et al. 2002, Lane et al. 2006) in placing members of the genus *Chroomonas* as a paraphyletic group leading into *Hemiselmis* (Fig. 1a). The *Hemiselmis* strains grouped in a tight cluster with strong support in all analyses, and sequences from two unidentified cryptomonad cultures (CCMP706 and CCMP1181) were also resolved within this clade, with *H. cryptochromatica* CCMP1181 branching as the sister taxon to other *Hemiselmis* strains and *H. pacifica* CCMP706 branching robustly with *H. virescens* PCC157 (Fig. 1a). Subsequent analyses, including only *Hemiselmis* species and more variable data that could not be unambiguously aligned in the larger data set, revealed several strongly supported clusters of sequences (Fig. 1b).

Hemiselmis rufescens, described by Parke (1949), is the type species of the genus, and the PCC maintains the authentic strain (PCC563), but this strain has not been considered in molecular investigations of cryptomonads before now. The only surviving culture from Butcher's 1967 monograph is also housed at PCC (*H. brunnescens* PCC14) and was investigated by Marin et al. (1998) but was obtained from the

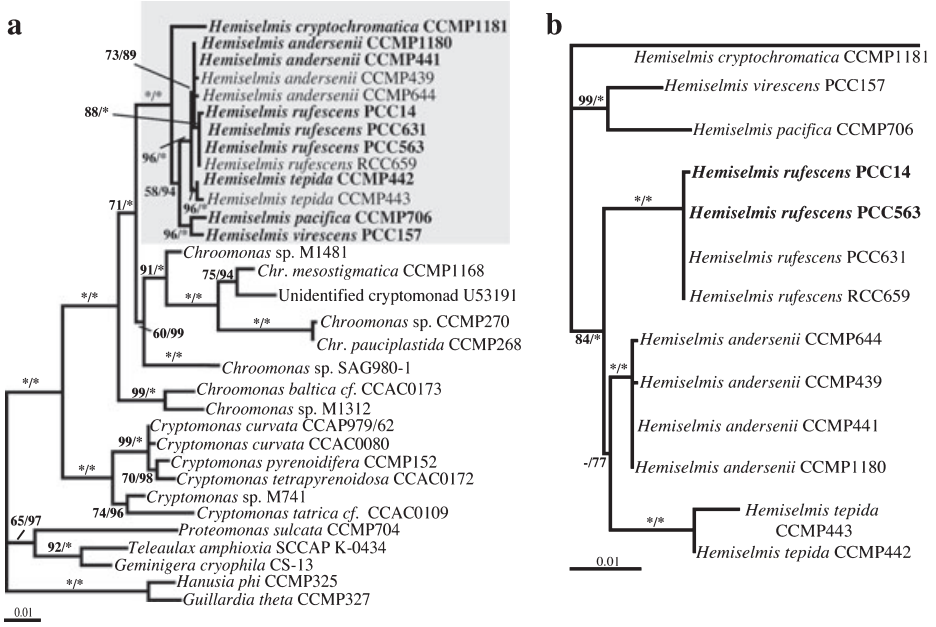

FIG. 1. Phylogenetic analysis of cryptomonad nucleomorph-encoded 18S rDNA sequences. (a) Maximum-likelihood (ML) tree of nucleomorph 18S rDNA sequences from members of *Hemiselmis*, *Chroomonas*, and select outgroup taxa. New sequences obtained in this study are in bold. (b) Unrooted ML phylogeny of the *Hemiselmis* subtree shown in the gray box in (a). Strains in bold are the authentic strains for *H. rufescens* (PCC563) and *H. brunnescens* (PCC14). Stars indicate bootstrap values and posterior probabilities of 100%, whereas "–" indicates support vales of <50%. Scale bars represent inferred number of nucleotide substitutions per site.

CCAP as a synonym strain (CCAP984/2). Our molecular data indicate that PCC563 and PCC14 have nearly identical nucleomorph chromosome structures (see below) and nucleomorph 18S sequences (only one difference in 1,675 bp), indicating that they represent members of the same species and should not be separated according to Butcher (1967).

In a previous publication (Lane and Archibald 2006), members of *Hemiselmis* were shown to have an unusual nucleomorph genome architecture because they lack copies of the 18S and 28S rDNA on the second of their three nucleomorph chromosomes. In order to determine if this feature is diagnostic of all *Hemiselmis* species, nucleomorph karyotype analysis was carried out for all strains that would grow to high enough density for PFGE analysis (2 L at 1–2 $\times$ 10^8 cells $\cdot$ L^{-1}). Nucleomorph genome size ranged from 560 to 600 kb in all the strains examined (Fig. 2). Strains with identical, or nearly identical, nucleomorph SSU rDNA sequences had identical nucleomorph genome karyotypes (Figs. 1b and 2). The two clades of red *Hemiselmis*, *H. andersenii* and *H. rufescens*, were clearly differentiated by their nucleomorph karyotypes, with *H. rufescens* containing a larger nucleomorph genome, particularly with regard to the size of chromosome II. The karyotype of *H. tepida* CCMP443 was similar to that of *H. andersenii* (though all *H. tepida* chromosomes were slightly smaller; Fig. 2), reinforcing the weakly resolved relationship between them in the phylogenetic analysis (Fig. 1b). Unfortunately, we were unable to grow CCMP442 to a high enough density for agarose plug formation and PFGE analysis, but the nucleomorph 18S sequence divergence between CCMP442 and 443 was only 0.5% and almost entirely localized in variable regions of the 18S rDNA. Strains that were resolved as sisters, but that had more significant sequence divergence (e.g., *H. virescens* PCC157 and *H. pacifica* CCMP706—1.7% 18S divergence), correlated with similar, but different, karyotypes. *H. rufescens* and *H. pacifica* share a very similar nucleomorph karyotype, but no other feature unites these taxa (see below).

Unexpectedly, *Hemiselmis cryptochromatica* CCMP1181 shows a significantly different karyotype pattern from the rest of the strains investigated. Whereas the ethidium bromide (EtBr)–stained gel shows three widely spaced chromosomes (data not shown), Southern hybridization using probes against the nucleomorph 5S rDNA gene did not produce a signal from the largest chromosome of *H. cryptochromatica*, but hybridized to the other two chromosomes (Fig. 2a). Probes designed to hybridize to the nucleomorph 18S and 28S rDNAs each produced a signal from only the largest and smallest chromosomes, similar to the other *Hemiselmis* strains. Additionally, the hybridization signal from the smallest *H. cryptochromatica* nucleomorph chromosome was approximately half as intense as that of the other chromosomes, using all three rDNA probes. This observation is similar to the pattern of 18S and 28S rDNA signal intensity from chromosomes I and III of all other *Hemiselmis* strains, which was interpreted as evidence for two copies of the 18S and 28S rDNA on chromosome I versus one copy on chromosome III (Lane and Archibald 2006). This finding has subsequently been confirmed from the complete nucleomorph genome sequence of *H. andersenii* CCMP644 (Lane et al. 2007).

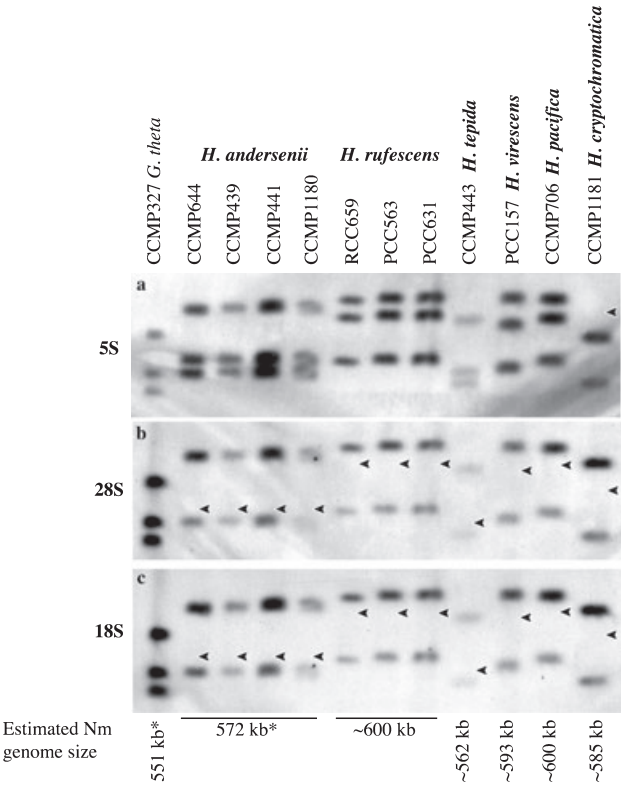

FIG. 2. Karyotype analysis of *Hemiselmis* nucleomorph genomes. Nucleomorph (Nm) chromosomes were separated by pulsed-field gel electrophoresis and probed in a Southern hybridization using the nucleomorph copy of the (a) 5S rDNA gene, (b) 28S rDNA, and (c) 18S rDNA. (a) The 5S gene is located at both ends of each chromosome in all strains except CCMP1181, where it is absent on chromosome I, suggesting that CCMP1181 shows an even greater degree of rDNA cistron loss than other members of *Hemiselmis* (see text). The black arrows indicate the position of ''missing'' chromosomes, inferred from the ethidium bromide (EtBr)–stained gel and the 5S rDNA Southern hybridization. The 28S (b) and 18S (c) rDNA are only found on chromosomes I and III in all *Hemiselmis* strains. Chromosomes missing portions of the rDNA cistron are a unique feature that unites members of this genus. ''*'' indicates exact genome size inferred from complete genome sequences. Strain names correspond to Table S1 (see the supplementary material).

Phycobiliprotein analysis. In agreement with earlier studies (Hill and Rowan 1989, Marin et al. 1998), we find only cryptomonad phycoerythrin (CrPE) with peak absorbance at 555 nm (CrPE555) in red *Hemiselmis* (*H. andersenii* and *H. rufescens*). Two cryptomonad phycocyanins (CrPCs) were found in our

blue-green *Hemiselmis* strains; *H. pacifica* and *H. tepida* contain a CrPC with an absorbance peak at 615 nm (CrPC615), whereas the CrPC within cells of *H. cryptochromatica* has an absorbance of 630 (CrPC630). CrPC630 was previously confined to members of *Chroomonas* (Hill and Rowan 1989, Marin et al. 1998, Deane et al. 2002), the outgroup to the *Hemiselmis* clade, and thus may represent retention of the ancestral pigmentation.

LM and EM. In light of the apparent discordance between the molecular results and the preexisting nomenclature for the genus, we revisited the morphological characteristics of the available strains using LM and EM. Observations made using phase contrast microscopy (Fig. 3) gave better results than DIC imaging for identifying cellular features in these small cells. Three marine CrPE555-containing *Hemiselmis* species have been previously described using LM and validly published—*H. amylosa*, *H. oculata*, and *H. rufescens* (Parke 1949, Butcher 1967). Only the authentic strain of *H. rufescens* remains for comparison to our *H. andersenii*, and the molecular data clearly separate these two taxa (Figs. 1 and 2). Comparisons to the descriptions of the remaining two species distinguished them from *H. andersenii* by distinctive attributes of their respective morphologies. *Hemiselmis amylifera* is unusual in being convex on both sides (Butcher 1967) and is generally more compressed than *H. andersenii* (2.5–3 μm vs. 3–5 μm). The size of *H. oculata* overlaps with *H. andersenii*, but features of the former species, such as a distinct lobe at the furrow and a pale yellowish region separating its two plastids, are inconsistent with the morphology of any of the four strains we include within *H. andersenii*.

Our *H. andersenii* strains (Fig. 3, e–g) were generally consistent in features such as the number of pyrenoids, plastid, and cellular arrangement, but differences in the gullet length, ejectisome arrangement, and prominence of the eyespot were apparent (Fig. 3). The most obvious of these differences was the refractive body (referred to as the maupas ovalis by some authors) of CCMP644 (Fig. 3g). This was a consistent feature of the culture, regardless of age, and upon close examination, could be observed in every cell. The large cells from the CCMP644 culture (Fig. 3g; see below) also tended to be slightly larger than those from either CCMP439 (Fig. 3e) or CCMP1180 (Fig. 3f). Refractive bodies were used by Butcher (1967) as a taxonomically useful feature (the only feature distinguishing between *H. virescens* and *H. simplex* in his scheme), but this character is not reflected in the clades recovered using molecular data presented here. In contrast to genetically similar cultures showing different morphologies, the opposite was also observed. The morphologies of *H. tepida* and

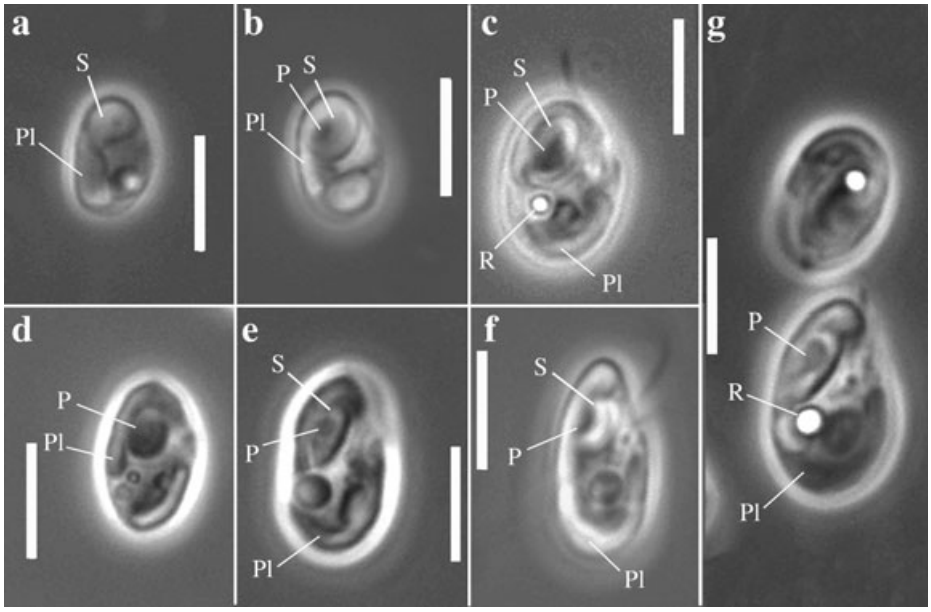

FIG. 3. Phase contrast light micrographs of *Hemiselmis* strains, all arranged so that their flagella emerge toward the upper right. (a) *Hemiselmis cryptochromatica* CCMP1181 (authentic strain), (b) *Hemiselmis tepida* CCMP443 (authentic strain), (c) *Hemiselmis pacifica* CCMP706 (authentic strain), (d) *Hemiselmis rufescens* PCC563 (authentic strain), (e) *Hemiselmis andersenii* CCMP439, (f) *Hemiselmis andersenii* CCMP1180, and (g) *Hemiselmis andersenii* CCMP644 (authentic strain). P = pyrenoid, Pl = plastid, R = refractive body, and S = starch. CCMP1181 (a) and CCMP443 (b) have a similar internal morphology despite being relatively phylogenetically divergent from one another (Fig. 1). The *H. andersenii* strains (e, f, and g) show similarities in their internal structure, generally with two pyrenoids (one with a starch coat), a parietal plastid, and a posterior nucleus. Differences in dorsoventral compression account for most of the differences in *H. andersenii* intracellular arrangement, such as the length of the gullet and position of the nucleus and ejectisomes. The refractive body of CCMP644 is the most obvious difference between the strains. Scale bars are all 5 μm.

H. cryptochromatica show considerable similarity in cellular structure, despite their genetic distance (Figs. 1 and 3, a, b).

Variability within strains serves to confuse further the interpretation of morphological features. CCMP644 exhibited two forms, similar to the situation previously described for *Proteomonas sulcata* Hill and Wetherbee (1986) and members of *Cryptomonas* (Hoef-Emden and Melkonian 2003). In *P. sulcata*, the two forms were distinct from one another in size, periplast configuration and structure, flagellar apparatus, and ploidy. Differences in the cell size and periplast type were also observed within the same cultures of *Cryptomonas* (Hoef-Emden and Melkonian 2003). Only cell size was measured (periplast and flagellar features were not examined) for CCMP644 (large cells = 7.5–8.5 μm × 4–5 μm; small cells = 5.5–6.5 μm × 3–4 μm), but the size difference between the two forms was nonoverlapping and similar to the size differences reported for the forms of *P. sulcata* (Hill and Wetherbee 1986), raising the possibility of variation associated with ploidy or life cycle further complicating morphological species description in *Hemiselmis*. We are confident that the two forms of CCMP644 are the same organism: we have recently sequenced both the nucleomorph and mitochondrial genomes of this strain (Lane et al. 2007) and found no ambiguities that would suggest the existence of two distinct strains in the same culture.

SEM revealed only slight differences among the cultures examined (Fig. 4). All strains showed the typical bean-shaped morphology described for members of *Hemiselmis*, with lateral insertion of the flagella. *Hemiselmis* lacks a furrow but maintains a small circular opening (vestibulum) to the gullet. The vestibulum opening was usually perpendicular to the long axis of the cell but was occasionally angled in all cultures examined. Cells from CCMP1181 tended to be obovoid to pyriform in dorsal view, having generally rounded ends (Fig. 4a). In contrast, CCMP706 (Fig. 4b), PCC14 (Fig. 4c), and CCMP644 (Fig. 4d) are acute at their anterior ends, but without a distinct tapering as in the posterior end of *Plagioselmis* (Novarino et al. 1994). As reported in previous studies (Santore 1977, 1982), fixation artifacts, such as collapse of the periplast, were persistent in critical-point-dried specimens, but underlying hexagonal plates could be observed in some cells with intact periplasts (data not shown).

In all samples, the anterior flagellum occasionally retained its mastigonemes (in two opposite rows, Fig. 4c) more often than the trailing flagellum, which was almost always naked or with small stubs in two rows (Fig. 4). The reason for this discrepancy is likely due to differences in mastigoneme attachment between the two flagella (Hibberd et al. 1971). A few remaining mastigonemes were occasionally observed on the trailing flagellum, suggesting that the stripped condition is an artifact of fixation rather than the natural condition. Ejectisomes were commonly discharged from cells and could be seen as a thread-like substance surrounding or covering some cells.

Whereas major differences between strains were not evident using SEM, evidence for cell size variation within cultures was prevalent in our electron micrographs (Fig. 5). Though cells of the small morphology were only a minor proportion of each

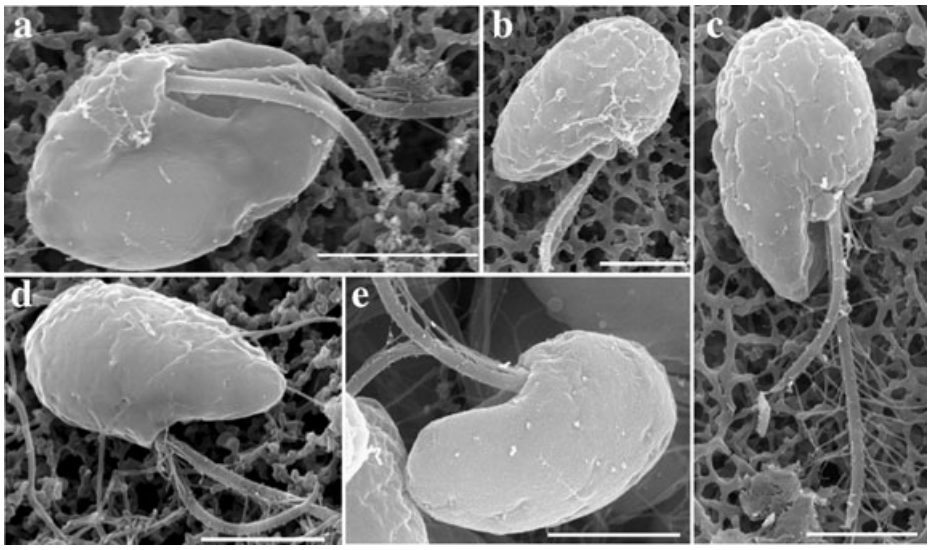

FIG. 4. Scanning electron micrographs of five *Hemiselmis* strains: (a) *H. cryptochromatica* CCMP1181, (b) *H. pacifica* CCMP706, (c) *H. rufescens* PCC14, (d) *H. andersenii* CCMP644, and (e) *H. virescens* PCC157. All cells were typical of the genus—reniform in lateral view, with the vestibulum about one-third the cell length from the anterior apex. The underlying hexagonal plates could be seen on some cells (a), and tubular mastigonemes (c) were more often present on the anterior flagellum than the trailing flagellum. Scale bars equal 5 μm.

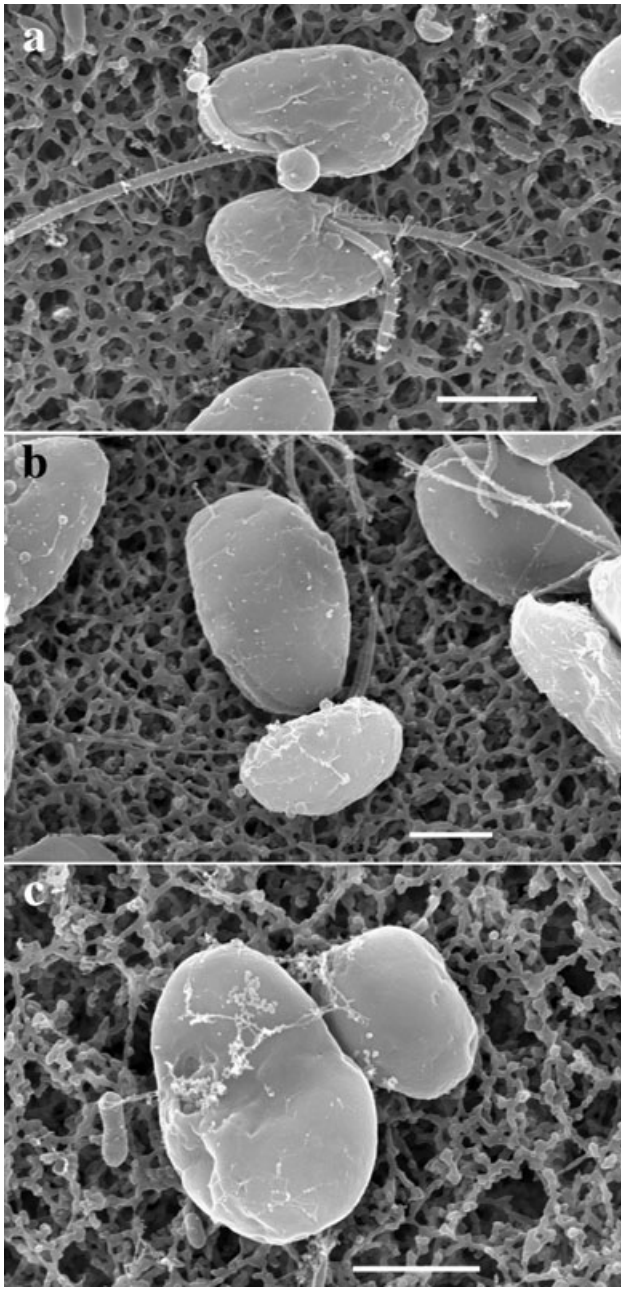

FIG. 5. Scanning electron micrographs of (a) *H. rufescens* PCC14, (b) *H. pacifica* CCMP706, and (c) *H. cryptochromatica* CCMP1181, showing examples of the large and small forms found in all cultures. In each culture, there was a minor proportion of cells that were significantly smaller than the majority. Scale bars are 2 μm.

culture in which they were found, there were examples in every culture examined.

DISCUSSION

Reliance on molecular characters for species diagnosis is still somewhat unusual, despite the massive amount of DNA sequence data being used in species-level studies. Whereas we have not included sequence data in the protologs above, GenBank accession numbers for the nucleomorph 18S rDNA sequences of these organisms are listed in Table S1. Additionally, we designate SEM stubs as types for our species and isotype DNA, which are available from the NYBG, with attempts at cryopreservation of the authentic strains ongoing at the CCMP (Robert Andersen, pers. com.).

The variability in cellular features within *Hemiselmis* species studied here is in stark contrast to the distinct clades of nearly identical molecular characters, which suggest discrete evolutionary units. Cellular features determined by LM or EM have been, by far, the characters of choice when distinguishing species of cryptomonads, with molecular methods only being used to test species boundaries recently in the genus *Cryptomonas* (Hoef-Emden and Melkonian 2003), where they found that species often take on two forms. The two forms of many *Cryptomonas* species had been separated into distinct genera, *Cryptomonas* and *Campylomonas*, based on their morphology, which has since been attributed to life-cycle stage. However, even though two distinct forms of many strains of *Cryptomonas* could be identified, one form was usually dominant in culture. Interestingly, if single cell or cyst isolates were used to start new cultures, the ratio of forms seen in culture could change dramatically (Hoef-Emden and Melkonian 2003). Based on these observations and the data presented here, it seems that the ability of cryptomonad species to take on more than a single form is widespread. Considering the morphological differences each species could display, Hoef-Emden and Melkonian (2003) concluded that it was not possible to describe *Cryptomonas* species on the basis of morphology alone, excluding molecular characters. Not surprisingly, we take that view here as well.

Despite not using sequence data in our species diagnoses, the nucleomorph karyotype data provided useful characters and were highly correlated with the sequence data. The PFGE analysis also provided independent confirmation of the 18S sequence data and an important control against a possible mix-up between our various cultures and their sequence because the DNA preparations for PCR and PFGE were done independently. Additionally, the correlation of the molecular characters with the geography and physiology of these strains (Table 1) further strengthens the argument for morphologically variable species. It remains possible that a detailed TEM study of within-versus-between species variability in cellular ultrastructure will identify morphological characters that are stable between the different strains and forms within each of these species. However, even if not, our inability to find consistent morphological features within each clade does not preclude them from being distinct species.

Butcher examined the culture of *H. rufescens* (PCC563) included here and compared it to his own collections. On the basis of his observations, he concluded that a new species should be created to accommodate the strain (now PCC14) he designated *H. brunnescens* (Butcher 1967, plates I and XIII), based almost entirely on cell size and color. We have examined PCC14 and confirmed that it does resemble Butcher's plates and is not a case of a culture that has been switched since his observations. Despite the differences in morphology, PCC563 and PCC14 are essentially identical at the molecular level, indicating morphological variability within species of *Hemiselmis*. Given the range of cell length in the description of *H. rufescens* (4.0–8.5 μm) and the relatively small size of *H. brunnescens* (5.0–5.5 μm), it is entirely possible that Butcher based his description of *H. brunnescens* on the small form of the strain, which clearly contains both a large and small form (Fig. 5a).

Even without recognizing the different forms within each strain of *Hemiselmis*, the value of LM as a tool to distinguish species in *Hemiselmis* has been previously questioned by Santore (1982), who used TEM to compare *H. brunnescens* and *H. virescens* to previous studies of *H. rufescens*. Santore pointed out that several features, deemed taxonomically useful by Butcher, are difficult or impossible to interpret correctly using LM techniques (ejectisome arrangement, organelle position, and gullet morphology) or are inconsistent in culture (eyespots, pyrenoids, and starch grains). At the level of TEM, Santore (1982) had difficulty separating *H. rufescens* and *H. brunnescens* from one another, finding only a slight difference in the supporting structure of the gullet and a lack of the ephemeral ejectisomes in a portion of the same structure. However, the amount of variability of these features between and within different strains of other species has not been assessed, making it difficult to interpret this observation. Therefore, Santore's data, coupled with the data presented here, cast doubt on many of Butcher's species, and, rather than legitimize those that were not validly published by Butcher, we choose to simply treat them as illegitimate names.

Particularly relevant to this argument is the variability in the morphology of different cultures that have nearly identical nucleomorph karyotypes and 18S rDNA sequences. Nucleomorph karyotype correlates strongly with the clades resolved based on sequence data, whereas morphology under LM varies within these clades. The *H. andersenii* clade is an excellent example of this phenomenon, as its members show obvious differences in cell size and morphology (Fig. 3, e–g), as well as small and large forms. At the moment, there are not enough data from multiple isolates of any blue-green *Hemiselmis* species to determine if the same amount of morphological diversity exists within the large forms of these species, but there are clearly different forms in blue-green *Hemiselmis* species as well (Fig. 5, b and c). However, there seems to be a similarity in morphology between the large forms of *H. cryptochromatica* and *H. tepida* (Fig. 3, a and b), with *H. tepida* being slightly larger. These species are some of the most distantly related in our trees and have different nucleomorph karyotypes and predicted temperature maxima (Table 1), enforcing the idea that describing cellular features with the light microscope is not sufficient to identify species of *Hemiselmis*.

Likewise, cell color has been suggested to be an unreliable character to divide cryptomonad taxa (Pringsheim 1944), despite Butcher's (1967) reliance on the feature to create the subgenera *Hemiselmis* (red members) and *Plagioselmis* (blue-green cells). However, whereas cell color may not reflect a strain's evolutionary history, absorbance of the phycobiliproteins of cryptomonads has been shown to correlate with molecular trees (Marin et al. 1998, Deane et al. 2002). Phycobiliprotein evolution is unusual in cryptomonads in that both phycoerythrin and phycocyanin pigments are derived from the red algal phycoerythrin (Sidler and Zuber 1988, Apt et al. 1995) of the cryptomonad plastid ancestor. The CrPCs are phycoerythrins that mimic the spectral characters of true phycocyanins by replacing phycoerythrobilin chromophores with phycocyanobilin chromophores (Sidler and Zuber 1988, Apt et al. 1995, Glazer and Wedemayer 1995). Therefore, the shift from cells that are blue-green in color to those that are red does not require massive changes in phycobiliproteins (which would be required to change from true phycocyanin to phycoerythrin), but only changes in the chromophores associated with the phycoerythrin protein complex.

The relatively minor cellular modifications required for cryptomonads to change their pigmentation are presumably responsible for the variation in cell color observed within *Hemiselmis*. However, not only are CrPC615 and CrPE555 unique to the genus, but *Hemiselmis* is the only cryptomonad genus that includes both CrPE and CrPC pigment types (Hill and Rowan 1989, Marin et al. 1998), making it an interesting test case for the evolution of pigments in cryptomonads. Our molecular data suggest that the evolution of CrPC615 and CrPE555 is recent, relative to the timescale of cryptomonad evolution (Fig. 1) and the distribution of phycobiliprotein types in other groups of cryptomonads (Marin et al. 1998), as both appear to have evolved since the split between *H. cryptochromatica* and all other *Hemiselmis* species. The sister relationship between the red *H. andersenii* and blue-green *H. tepida* to the exclusion of *H. rufescens* is not well supported in our phylogenetic analyses, but the nucleomorph karyotypes of *H. andersenii* and *H. tepida* are similar to one another and distinct from *H. rufescens*. If the resolved relationship reflects the evolution of this group, then at least two pigment changes have

occurred in *Hemiselmis* in addition to the initial switch from the ancestral CrPC630; either from CrPC615 to CrPE555 between the *H. virescens/ H. pacifica* clade and *H. rufescens*, followed by a switch from CrPE555 to CrPC615 in the ancestor of *H. tepida*, or two independent changes from CrPC615 to CrPE555 in each of the red species, *H. andersenii* and *H. rufescens*.

Hemiselmis andersenii and *H. rufescens* represent the only known reversal(s) from CrPC to CrPE pigments in the cryptomonads, and the reacquisition of red chromophores is presumably the reason that red *Hemiselmis* species contain a unique phycoerythrin (Clay and Kugrens 1999). However, even within *H. andersenii*, there is obvious variation in the proportion of pigments within each species, resulting in substantial cell color differences—from a deep red (CCMP439) to orange/ brown (CCMP1180). Additionally, *H. cryptochromatica* (CCMP1181) shows almost no coloration in our cultures (even when grown in high density and shaded conditions), which is considerably different than the dark green displayed by most *Chroomonas* species, the nearest outgroup, as well as *H. virescens* and *H. pacifica*. Our data support the conclusion that cell color is not a reliable character to separate genera or split up species into subgenera as in Butcher's taxonomic scheme (Butcher 1967).

Both the red *H. andersenii* and blue-green *H. tepida* were collected from the warm waters of the Gulf of Mexico and Gulf Stream, and their maximum growth temperature (>25°C) presumably reflects an adaptation to this environment. The remaining *Hemiselmis* species included in our analyses were collected from cooler water in both the Pacific and Atlantic and do not survive in the lab much above 20°C, suggesting that *H. andersenii* and *H. tepida* have physiological adaptations separating them from the other taxa included in this study and further supporting a relationship between these taxa. Only one strain of *Hemiselmis* collected outside of the northern Atlantic (*H. pacifica*, CCMP706) is currently recognized, but as sampling increases worldwide, the number of species in this genus of cryptomonads is also bound to increase.

With the morphological variability within *Hemiselmis* species, it seems clear that rapid and accurate identification by LM is not possible. The combination of microscopy and collection location may provide a loose indication of taxonomic affinity in the absence of molecular data. Undoubtedly, there are more *Hemiselmis* species than just those included here, but of those currently available in public culture collections, there are no sister species that share the same combination of cell color, geography, and growth temperature maximum (Table 1). However, DNA sequencing is currently the fastest way to unambiguously classify these tiny flagellates.

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Apt, K. E., Collier, J. L. & Grossman, A. R. 1995. Evolution of the phycobiliproteins. *J. Mol. Biol.* 248:79–96.

Archibald, J. M. 2007. Nucleomorph genomes: structure, function, origin and evolution. *Bioessays* 29:392–402.

Archibald, J. M. & Keeling, P. J. 2005. On the origin and evolution of plastids. *In* Saap, J. [Ed.] *Microbial Phylogeny and Evolution.* Oxford University Press, New York, pp. 238–60.

Butcher, R. W. 1967. An introductory account of the smaller algae of British costal waters. *Fish. Investig. Lond. Ser.* IV:1–54.

Clay, B. L. & Kugrens, P. 1999. Characterization of *Hemiselmis amylosa* sp. nov. and phylogenetic placement of the blue-green cryptomonads *H. amylosa* and *Falcomonas daucoides*. *Protist* 150:297–310.

Deane, J. A., Strachan, I. M., Saunders, G. W., Hill, D. R. A. & McFadden, G. I. 2002. Cryptomonad evolution: nuclear 18S rDNA phylogeny versus cell morphology and pigmentation. *J. Phycol.* 38:1236–44.

Delwiche, C. F. 1999. Tracing the thread of plastid diversity through the tapestry of life. *Am. Nat.* 154(Suppl.):S164–77.

Douglas, S., Zauner, S., Fraunholz, M., Beaton, M., Penny, S., Deng, L. T., Wu, X., Reith, M., Cavalier-Smith, T. & Maier, U. G. 2001. The highly reduced genome of an enslaved algal nucleus. *Nature* 410:1091–6.

Droop, M. R. 1955. Some new supra-littoral protista. *J. Mar. Biol. Assoc. U. K.* 34:233–54.

Eschbach, S., Hofmann, C. J., Maier, U.-G., Sitte, P. & Hansmann, P. 1991. A eukaryotic genome of 660 kb: electrophoretic karyotype of nucleomorph and cell nucleus of the cryptomonad alga, *Pyrenomonas salina*. *Nucleic Acids Res.* 19:1779–81.

Gilson, P. R., Su, V., Slamovits, C. H., Reith, M. E., Keeling, P. J. & McFadden, G. I. 2006. Complete nucleotide sequence of the chlorarachniophyte nucleomorph: nature's smallest nucleus. *Proc. Natl. Acad. Sci. U. S. A.* 103:9566–71.

Glazer, A. N. & Wedemayer, G. J. 1995. Cryptomonad biliproteins – an evolutionary perspective. *Photosynth. Res.* 46:93–105.

Guindon, S. & Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52:696–704.

Hibberd, D. J., Greenwood, A. D. & Griffiths, H. B. 1971. Observations on the ultrastructure of the flagella and periplast in the Cryptophyceae. *Br. Phycol. J.* 6:61–72.

Hill, D. R. A. & Rowan, K. S. 1989. The biliproteins of the Cryptophyceae. *Phycologia* 28:455–63.

Hill, D. R. A. & Wetherbee, R. 1986. *Proteomonas sulcata* gen. et sp. nov. (Cryptophyceae), a cryptomonad with two morphologically distinct and alternating forms. *Phycologia* 25:521–43.

Hoef-Emden, K., Marin, B. & Melkonian, M. 2002. Nuclear and nucleomorph SSU rDNA phylogeny in the Cryptophyta and the evolution of cryptophyte diversity. *J. Mol. Evol.* 55:161–79.

Hoef-Emden, K. & Melkonian, M. 2003. Revision of the genus *Cryptomonas* (Cryptophyceae): a combination of molecular phylogeny and morphology provides insights into a long-hidden dimorphism. *Protist* 154:371–409.

Huelsenbeck, J. P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754.

Keeling, P. J. 2004. Diversity and evolutionary history of plastids and their hosts. *Am. J. Bot.* 91:1481–93.

Kugrens, P., Lee, R. E. & Hill, D. R. A. 2000. Order Cryptomonadida Senn, 1900. *In* Lee, J. J., Leedale, G. F. & Bradbury, P. [Eds.] *An Illustrated Guide to the Protozoa.* Society of Protozoologists, Lawrence, Kansas, pp. 1111–25.

Lane, C. E. & Archibald, J. M. 2006. Novel nucleomorph genome architecture in the cryptomonad genus *Hemiselmis. J. Eukaryot. Microbiol.* 53:515–21.

Lane, C. E., Khan, H., MacKinnon, M., Fong, A., Theophilou, S. & Archibald, J. M. 2006. Insight into the diversity and evolution of the cryptomonad nucleomorph genome. *Mol. Biol. Evol.* 23:856–65.

Lane, C. E., van den Heuvel, K., Kozera, C., Curtis, B. A., Parsons, B., Bowman, S. & Archibald, J. M. 2007. Nucleomorph genome of *Hemiselmis andersenii* reveals complete intron loss and compaction as a driver of protein structure and function. *Proc. Natl. Acad. Sci. U. S. A.* 104:19908–13.

Marin, B., Klingberg, M. & Melkonian, M. 1998. Phylogenetic relationships among the Cryptophyta: analyses of nuclear-encoded SSU rRNA sequences support the monophyly of extant plastid-containing lineages. *Protist* 149:265–76.

Novarino, G., Lucas, I. A. N. & Morrall, S. 1994. Observations on the genus *Plagioselmis* (Cryptophyceae). *Cryptogam. Algol.* 15:87–107.

Parke, M. 1949. Studies on marine flagellates. *J. Mar. Biol. Assoc. U. K.* 28:255–88.

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–8.

Pringsheim, E. G. 1944. Some aspects of taxonomy in the Cryptophyceae. *New Phytol.* 43:367–401.

Rensing, S. A., Goddemeier, M., Hofmann, C. J. & Maier, U.-G. 1994. The presence of a nucleomorph hsp70 gene is a common feature of Cryptophyta and Chlorarachniophyta. *Curr. Genet.* 26:451–5.

Santore, U. J. 1977. Scanning electron microscopy and comparative micromorphology of the periplast of *Hemiselmis rufescens*, *Chroomonas* sp., *Chroomonas salina* and members of the genus *Cryptomonas* (Cryptophyceae). *Br. Phycol. J.* 12:255–70.

Santore, U. J. 1982. The ultrastructure of *Hemiselmis brunnescens* and *Hemiselmis virescens* with additional observations on *Hemiselmis rufescens* and comments on the Hemiselmidaceae as a natural group of the Cryptophyceae. *Br. Phycol. J.* 17:81–99.

Sidler, W. & Zuber, H. 1988. Structural and phylogenetic relationships of phycoerythrins from cyanobacteria, red algae and Cryptophyceae. *In* Scheer, H. & Schneider, S. [Eds.] *Photosynthetic Light-Harvesting Systems.* Walter de Gruyter & Co., Berlin, pp. 49–60.

Silva, P. C. 1980. Names of classes and families of living algae: with special reference to their use in the Index Nominum Genericorum (Plantarum). *Regnum Veg.* 103:1–156.

Swofford, D. L. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods),* Version 4.0b10 PPC. Sinauer Associates, Sunderland, Massachusetts.

Zauner, S., Fraunholz, M., Wastl, J., Penny, S., Beaton, M., Cavalier-Smith, T., Maier, U.-G. & Douglas, S. 2000. Chloroplast protein and centrosomal genes, a novel tRNA intron, and odd telomeres in an unusually compact eukaryotic genome – the cryptomonad nucleomorph. *Proc. Natl. Acad. Sci. U. S. A.* 97:247–60.

Supplementary Material

The following supplementary material is available for this article:

Table S1. Species used for phylogenetic analysis. The strain identification numbers are listed for each culture used, as well as the GenBank accession number if the data have been previously published. Nucleomorph 18S rDNA sequences obtained in this study are in bold type.

This material is available as part of the online article from:http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00486.x.

(This link will take you to the article abstract.)

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