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Hanusia phi gen. et sp. nov. (Cryptophyceae): characterization of '*Cryptomonas* sp. Φ'

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Cryptomonas sp. Φ is an undescribed cryptomonad used for many studies into the endosymbiotic origin of plastids. *Cryptomonas* sp. Φ was characterized using electron microscopy, DNA sequencing and karyotyping. DNA sequence data show that *Cryptomonas* sp. Φ has been confused with the related cryptomonad, *Guillardia theta*, which is the organism actually used in most published studies on cryptomonad endosymbiosis to date. It is proposed that the strain *Cryptomonas* sp. Φ be recognized as a distinct species under the name *Hanusia phi*. Although *Hanusia phi* shares molecular and karyotypic features with *G. theta*, it cannot be assigned to *Guillardia* because it does not fit the morphological description of this genus. *H. phi* differs from *G. theta* in periplast structure, configuration of the furrow-gullet system, and cell size. Morphologically, however, *H. phi* is very similar to *Teleaulax acuta*, *Teleaulax merimbula* and the diplomorph of *Proteomonas sulcata*.

Key words: cryptomonad, *Cryptomonas* sp. Φ, *Hanusia phi*, nucleomorph

Introduction

In 1963 Guillard reported the isolation of a cryptomonad he designated 'flagellate Φ' (Guillard, 1963). Gillot & Gibbs (1983) regarded flagellate Φ as a species of *Cryptomonas*, and referred to it as *Cryptomonas* sp. Φ. Since then, *Cryptomonas* sp. Φ has become a model organism for molecular investigation of the endosymbiotic origin of the plastids in cryptomonads (e.g. Douglas, 1988, 1991; Douglas & Dunford, 1989, 1990*a*, *b*; Douglas & Turner, 1991; Douglas *et al*., 1990, 1991; Wang & Liu, 1991; McFadden *et al*., 1994*a*, *b*; Rensing *et al*., 1994; Reith & Douglas, 1990). Cryptomonads acquired plastids by secondary endosymbiosis, a process in which a eukaryotic alga (itself the product of primary endosymbiosis between a photosynthetic prokaryote and a eukaryotic host) was engulfed and retained by a eukaryotic phagotroph (see reviews by McFadden & Gilson, 1995, and McFadden *et al*., 1997).

Cryptomonas sp. Φ has not been formally described as a species, so we undertook a morphological and molecular characterization for its taxonomic description. During our investigations, it became apparent that *Cryptomonas* sp. Φ has been confused with *Guillardia theta* Hill *et* Wetherbee (Hill & Wetherbee, 1990). Many studies of endosymbiosis in cryptomonads have inadvertently used *G. theta* instead of *Cryptomonas* sp. Φ. Here, *Cryptomonas* sp. Φ is described and compared with *G. theta* and a formal name proposed.

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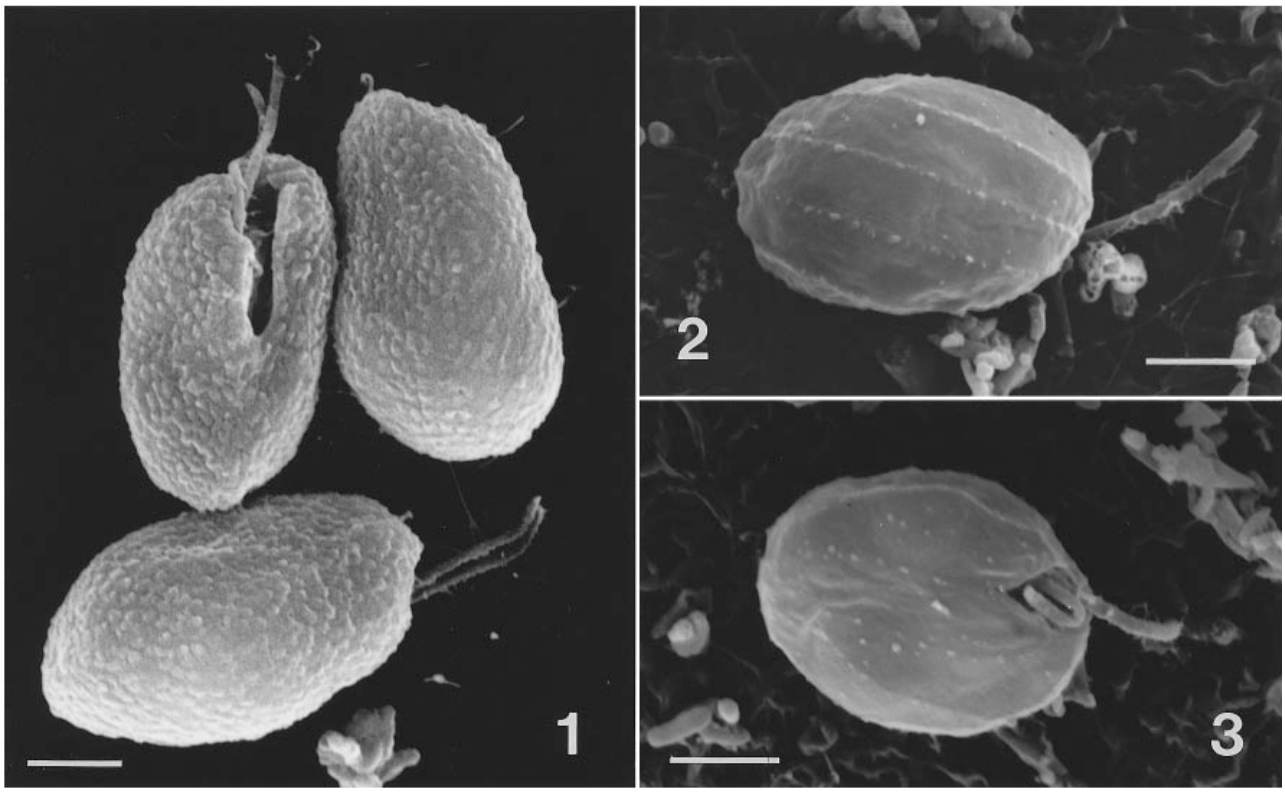
Materials and methods

Cryptomonas sp. Φ (CCMP 325) and *Guillardia theta* (CCMP 327) were obtained from the Bigelow Culture Collection of Marine Phytoplankton and maintained in H/2 seawater medium (McLachlan, 1973) in a south-facing window at 20 °C.

For scanning electron microscopy, cells in culture medium were fixed for 2 h by addition of an equal volume of 2% osmium tetroxide in seawater and filtered onto a 2 μm pore size isopore filter (Millipore). Filters and attached cells were washed with distilled water, dehydrated in ethanol, critical-point-dried and coated with gold. For transmission electron microscopy, cells were harvested by centrifugation, fixed for 1 h with 2% glutaraldehyde in 0·05 M sodium cacodylate buffer pH 7·5 plus 0·4 M sucrose, post-fixed in 1% osmium tetroxide in 0·05 M sodium cacodylate buffer, dehydrated in ethanol, and embedded in Spurr's resin (Spurr, 1969). Embedded cells were thin sectioned and stained with uranyl acetate and lead citrate. Cells were prepared for freeze fracture as reported by Hill & Wetherbee (1986).

To prepare cells for pulsed field gel electrophoresis (PFGE), they were harvested by centrifugation and resuspended in plug buffer (10 mM Tris/HCl pH 7.5, 100 mM Na_2EDTA, and 100 mM NaCl). Resuspended cells were mixed with an equal volume of 1.2% low-gelling-temperature agarose in plug buffer at 37 °C and pipetted into chilled plug moulds. Once solid, plugs were digested for 48 h at 50 °C in two changes of 10 mM Tris/HCl pH 7.5, 400 mM Na_2EDTA, and 10 mg ml^{-1}



Figs 1–3. External appearance of *Cryptomonas* sp. **Φ** (CCMP 325) and *Guillardia theta* (CCMP 327). Fig. 1. Cells of *Cryptomonas* sp. **Φ** showing the periplast, furrow and flagella. Figs 2 , 3. *G. theta*. Fig. 2. Detail of longitudinally oriented periplast plates. Fig. 3. Gullet opening and flagella. Scale bars represent 2 μm.

Pronase E (Sigma P-5147). Plugs were stored in 10 mM Tris/HCl pH 7.5 at 4 °C and electrophoresed in 1% agarose, 0.5 × TBE buffer using a BioRad CHEF DRIII apparatus. The following electrophoresis conditions were used: 65 h run, 14 °C, 30 s decreasing to 10 s pulse change interval and 4.1 V cm^{-1}.

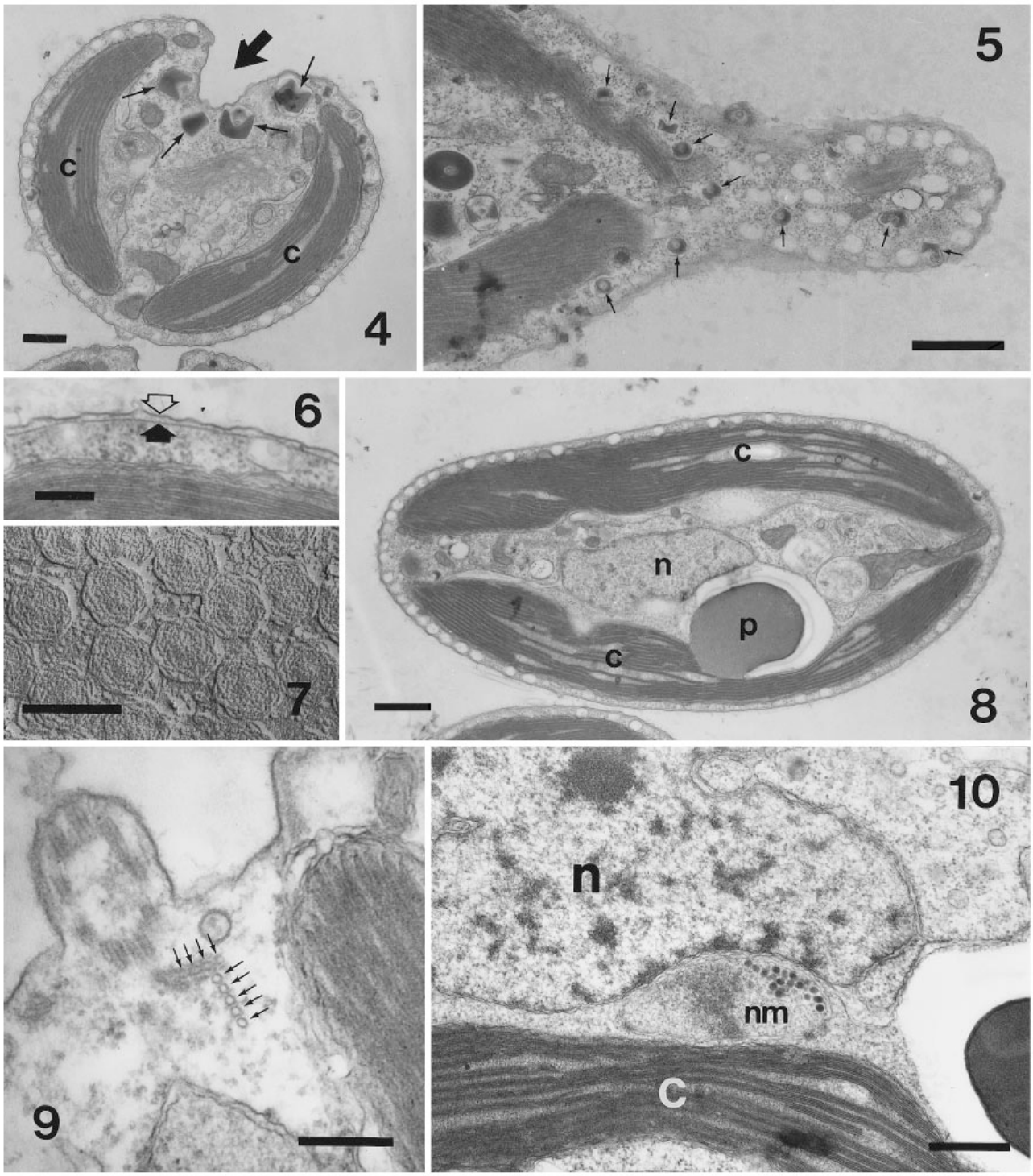
The 18S ribosomal RNA (rRNA) gene from the CCMP 325 host cell nucleus was amplified using the polymerase chain reaction (PCR) and the two universal eukaryotic 18S primers TACCTGGTGGATCCTGCCAG (FAD3) and TGATCCTTCTGCAGGTTCACCTAC (FAD4). *Cryptomonas* sp. **Φ** and *G. theta* nucleomorph 18S rRNA genes were amplified using the FAD4 primer in conjunction with a primer specific for the cryptomonad nucleomorph 18S gene (Cavalier-Smith *et al.*, 1996). PCR reactions were electrophoresed in 0.8% agarose, and 18S rRNA gene PCR products excised then purified using Prep-a-Gene™ (BioRad). PCR products were restriction-digested and cloned using plasmid vectors. Cloned PCR products were sequenced using universal 18S rRNA and plasmid-specific primers in conjunction with dye-labelled dideoxy terminators (ABI) on an ABI 373a Automated Sequencer. Sequences were edited and assembled using Sequencher 3.0 (Gene Codes, Michigan) and aligned with ClustalW (Thompson *et al.*, 1994).

The complete nuclear (X57162) and nucleomorph (X57008) 18S rRNA sequences attributed to *Cryptomonas* sp. **Φ** by Douglas *et al.* (1991) were obtained from GenBank.

Results

Morphology

The general morphology of *Cryptomonas* sp. **Φ** has been reported by Gillot & Gibbs (1983) and our results are in close agreement with their data. *Cryptomonas* sp. **Φ** (Fig. 1) is easily distinguished from *Guillardia theta* (Figs 2, 3) when viewed by scanning electron microscopy. Cells of *Cryptomonas* sp. **Φ** (CCMP 325) are approximately ovoid, 5–10 μm in length, 3–5 μm wide and have two flagella anchored at the anterior end of a furrow (Fig. 1). The furrow runs about half the length of the cell and is lined with rows of large ejectisomes (Figs 1, 4). Cells from a rapidly growing culture may have a narrow tail at the posterior end of the cell (Fig. 5). The periplast is of the sheet type and the surface component is covered with a dense mat of rosulate scales (Figs 6, 7). These scales are approximately 130 nm in diameter and are composed of densely packed granular material surrounding a slightly raised axis (Fig. 7). Many small ejectisomes are found in vesicles underneath the periplast, particularly at the posterior of the cell (Fig. 5). Each interphase cell has one orange-red plastid and a pyrenoid without traversing thylakoids that is surrounded by a starch sheath (Fig. 8). Cells sometimes have two plastids (not shown), a condition that is assumed to exist just prior to cell division. The predominant biliprotein in the plastid is Cr-phycoerythrin 545 (cryptomonad phycoerythrin type I). An ascending flagellar root complex (Gillot & Gibbs,



Figs 4–10. Ultrastructure of *Cryptomonas* sp. **Φ** (CCMP 325). c, plastid; n, nucleus. Fig. 4. Cross-section of cell with four large ejectisomes (small arrows) surrounding furrow (large arrow). Fig. 5. Posterior region of cell with many small ejectisomes (arrows) in vacuoles beneath the periplast. Fig. 6. Cross-section through the periplast showing the inner (arrow) and outer (open arrow) components. Fig. 7. Freeze-etch image of scales on the surface periplast component. Fig. 8. Longitudinal section showing internal architecture. The pyrenoid (p) is surrounded by a starch sheath. Fig. 9. The ascending microtubular flagellar root complex displaying 2 component roots. Arrows show individual microtubules. Fig. 10. Detail of the nucleomorph (nm) and its position within the cell. Note electron-dense particles and nucleolus. Scale bars represent: Figs 4 and 10, 500 nm; Figs 5 and 8, 1 μm; Figs 6, 7 and 9, 200 nm.

1983) consists of 2 microtubular roots (Fig. 9). Gillot & Gibbs (1983) provide a detailed description of the flagellar apparatus. The nucleomorph is free of the pyrenoid and contains a number of electron-dense granules together with a fibrillogranular nucleolus (Fig. 10). Cells usually exist as single, motile monads and do not form palmelloid colonies.

Guillardia theta (CCMP 327) (Figs 2, 3) was as described by Hill & Wetherbee (1990). In comparison with *Cryptomonas* sp. **Φ**, *G. theta* is shorter, rounder and has a gullet

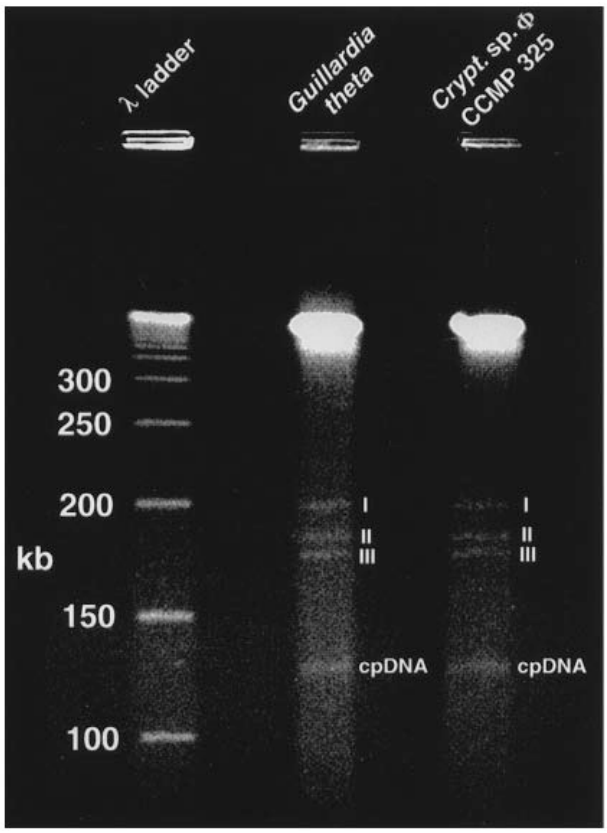


Fig. 11. Pulsed field gel of *Guillardia theta* and *Cryptomonas* sp. **Φ** (CCMP 325) showing nucleomorph chromosomes I, II and III, plastid DNA (cpDNA) and a bright band of nuclear chromosomes.

but no furrow (Fig. 3). The cell surface of *G. theta* consists of longitudinally orientated plates that form a raised rib where they meet (Fig. 2).

Nucleomorph karyotyping

The nucleomorph karyotype of *Cryptomonas* sp. **Φ** as resolved by PFGE is indistinguishable from that of *G. theta* (Fig. 11). In both species, nucleomorph chromosomes are approximately 175, 180 and 195 kb. A 130 kb band representing the plastid DNA (McFadden *et al.*, 1994*a*) is also resolved. Nuclear chromosomes are too large to be resolved under these conditions and migrate as a single band in both species (Fig. 11).

Molecular analysis

The 18S rRNA genes amplified from the nucleus (GenBank XU53126) and nucleomorph (GenBank XU531254) of *Cryptomonas* sp. **Φ** (CCMP 325) were 1728 bp and 2021 bp respectively excluding primers. When the sequences of these genes were aligned with the corresponding GenBank entries nominally for *Cryptomonas* sp. **Φ** (Douglas *et al.*, 1991), there were 14 mismatches along the length of the nuclear genes and 264 between the nucleomorph genes. To test whether the Douglas *et al.* (1991) sequence was actually from *G. theta* rather than from *Cryptomonas* sp. **Φ**, we sequenced a variable region of the 18S rRNA from *G. theta* (CCMP 327). Comparison of the sequence from this 463 bp *Pst*I nucleomorph fragment of *G. theta* (CCMP 327) (GenBank AF047376) with the corresponding nucleomorph sequence lodged in GenBank under the name *Cryptomonas* sp. **Φ** revealed near identity, with only three mismatches (Fig. 12). Conversely, within the same region, the real *Cryptomonas* sp. **Φ** (CCMP 325) and the supposed *Cryptomonas* sp. **Φ** (Douglas *et al.*, 1991) have 32 mismatches (Fig. 12).

```
G.theta..... CTGCAGCGGG ATTTATTAGA TCAAAAACCA ATCGCATCTC CCTGC--CAT GGGC---GAG TGCGTGGCGG GATGAATCAT
Douglas et al. CTGCAGCGGG ATTTATTAGA TCAAAAACCA ATCGCATCTC CCTTC--CAT GGGC---GAG TGCGTGGCGG GATGAATCAT
CCMP 325..... GTGCAGCGGG ATTTATTAGA TCAAAAACCA ATCGCAACTC CCTTCTTCAA AGGTAGGGGA TGCGTGGCGG GATGAATCAT
             #                                        #       * ##  # #  #### ##

                                                                                                    160
G.theta..... AATAACTTTT TTTTTTTTTT TTTTTCCGAA CCGTATGGCT TCCAAT-TCT CATGGCCGGC GGTAAATCAT TCAAATTTCT
Douglas et al. AATAACTTTT TTTTTTTTTT TTTT-CCGAA CCGTATGGCT TCCAAT-TCT CATGGCCGGC GGTAAATCAT TCAAATTTCT
CCMP 325..... AATAACTAAT CTTTTTT--- -----CCAAA CCGTATGGCT TTTATCATTT CATGGCCGAC GGTAAATCAT TCAAATTTCT
                 ##  #       ### ####*  #                ## ### #           #

                                                                                                    240
G.theta..... GCCCTATCAA CTTTCGATGG TTGGGTAGTG GCCAACCATG GTGTTGACGG GTACGGGGAA TTAGGGTTCG ATTCCGGAGA
Douglas et al. GCCCTATCAA CTTTCGATGG TTGGGTAGTG GCCAACCATG GTGTTGACGG GTACGGGGAA TTAGGGTTCG ATTCCGGAGA
CCMP 325..... GCCCTATCAA CTTTCGATGG TTGGGTAGTG GCCAACCATG GTGTTGACGG GTACGGGGAA TTAGGGTTCG ATTCCGGAGA

                                                                                                    320
G.theta..... GGGAGCCTGA GAGATGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGAAA ATTACCCAAT CCTGATTCAG GGAGGTAGCG
Douglas et al. GGGAGCCTGA GAGATGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGAAA ATTACCCAAT CCTGATTCAG GGAGGTAGCG
CCMP 325..... GGGAGCCTGA GAGATGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGAAA ATTACCCAAT CCTGATTCAG GGAGGTAGCG

                                                                                                    400
G.theta..... ACAAGAAATA ACGGTAGTGG ACTCGTTCGA GTCTGCTCAT CGGAATGAGG ATAGTTTACA CCCCTTCTCG AGGATCCATT
Douglas et al. ACAAGAAATA ACGGTAGT-G ACTCGTTCGA GTCTGCTCAT CGGAATGAGG ATAGTTTACA CCCCTTCTCG AGGATCCATT
CCMP 325..... ACAAGAAATA ACGGTAGTGG GCTCGTTCGA GTCTGCTCAT CGGAATGAGG ATAGTTTACA CCCCTTCTCG AGGATCCATT
                           *  #

G.theta..... GGAGGGCAAG TCTGGTGCC AGCAGCCGCGG TAACTCCAGC TCCAATAGCG TATACTAAAG TTGCTGCAG
Douglas et al. GGAGGGCAAG TCTGGTGCC AGCAGCCGCGG TAACTCCAGC TCCAATAGCG TATACTAAAG TTGCTGCAG
CCMP 325..... GGAGGGCAAG TCTGGTGCC AGCAGCCGCGG TAACTCCAGC T-CAATAGCG TATACTAAAG TTGCTGCAG
                                                      #
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Fig. 12. Alignment of a region of the nucleomorph 18S ribosomal RNA genes from *Guillardia theta* (CCMP 327), the cryptomonad used by Douglas *et al.* (1991) and *Cryptomonas* sp. **Φ** (CCMP 325). Bases are in bold at positions where all three sequences are not the same. # indicates positions where the sequence of Douglas *et al.* (1991) does not match CCMP 325. * indicates a position where the sequences of Douglas *et al.* and *G. theta* do not match. Dashes show where gaps have been inserted to facilitate alignment.

Diagnoses

Hanusia, genus novum

Cryptomonadales Novarino et Lucas; monadibus perpetuo natantibus, marinis; vestibulo subapicali in sulcum ad medium retrorsum ducenti; periplasto lamellis nullis, lamina interiore poris qui ejectisomatibus commodant regulatim interrupta, exteriore continua cum squamis rosulatis imbricatis et fibrillis crassis immixtis, sine fascia midiventrali; chromatophoro unico, pigmento Cr-phycoerythrino 545, pyrenoide sine thylacoidibus perducentibus; nucleomorphe unica dorsali inter pyrenoidem nucleumque: ut morphologia proxime *Teleaulacem* Hill atque *Proteomonadis* Hill et Wetherbee diploideomorphem differt tamen XVIIIS rRNA structura.

Species typica: *Hanusia phi*, sp. nov.

Cryptomonadales Novarino & Lucas; perpetually swimming, marine; vestibulum subapical, posteriorly drawn into a furrow running about halfway down the length of the cell; without periplast plates, an inner sheet interrupted regularly with pores to accommodate ejectisomes, the exterior a continuous layer of rosulate scales with some intermixed coarse fibrils, without a mid-ventral band; single chromatophore with Cr-phycoerythrin 545 pigment (cryptomonad phycoerythrin type I); single pyrenoid without traversing thylakoids; single nucleomorph dorsal between the pyrenoid and nucleus: similar in morphology to *Teleaulax* Hill and the *Proteomonas* Hill & Wetherbee diplomorph but differing in 18S ribosomal RNA gene sequence.

Type species: *Hanusia phi*, sp. nov.

Etymology: *Hanusia*. Named in memory of the phycologist Hanŭs Ettl.

Hanusia phi Deane, species nova

5–10 μm longis, 3–5 μm latis, leviter compressis, aspectu laterali ex elliptica obovatum; plerumque cum quattuor plusve seriebus ejectisomatum; pyrenoide vaginam amyli perfectam facienti.

Holotypus: Figura 13.

5–10 μm long, 3–5 μm wide, slightly compressed, narrowly elliptic to obovate in lateral view; usually with 4 or more rows of ejectisomes; pyrenoid with a continuous starch sheath.

Holotype: Fig. 13.

Observations: *Hanusia phi*, previously known as *Cryptomonas* sp. Φ, is a new member of the Cryptomonadales (Novarino & Lucas, 1993), very close in morphology to *Teleaulax* and the diplomorph of *Proteomonas*, but distinct in its 18S rRNA gene sequence, which is similar to that of *G. theta* (Hill & Saunders, unpublished data); differing from *G. theta*, however, in periplast structure, configuration of the furrow-gullet system, and cell size.

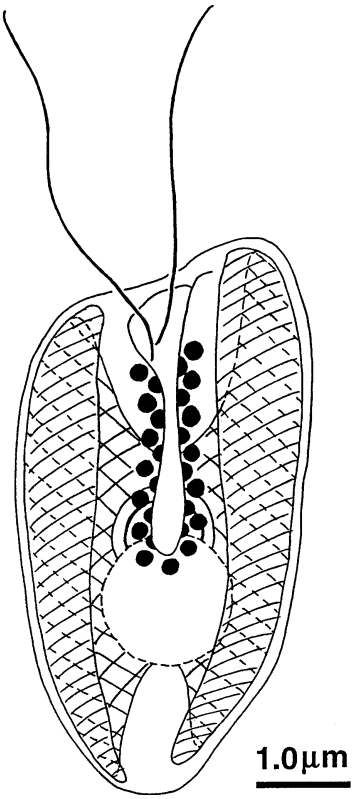

Fig. 13. Drawing of *Hanusia phi* showing features visible by light microscopy.

Discussion

The external appearance and ultrastructure of CCMP 325 are as described for *Hanusia phi* (*Cryptomonas* sp. Φ) by Gillot & Gibbs (1983). *Hanusia phi* is easily distinguished from *Guillardia theta* on the basis of size, shape and periplast type.

18S rRNA phylogeny, using either nuclear or nucleomorph genes, shows that *Hanusia phi* is clearly the sister taxon to *G. theta* (Cavalier-Smith *et al.*, 1996). A close relationship between *Hanusia phi* and *G. theta* is also supported by these species having identical nucleomorph karyotypes (Fig. 11). This seems particularly striking since Rensing *et al.* (1994) resolved the nucleomorph chromosomes of six cryptomonad species and none was found to share the same nucleomorph karyotype.

The circumscription of *Cryptomonas* has recently been revised and *Hanusia phi*, which had previously been referred to this genus, does not conform to the altered description (Hill, 1991). Although *Hanusia phi* shares molecular and karyotypic features with *G. theta*, it cannot be assigned to *Guillardia* because it does not fit the physical description of this genus (Hill & Wetherbee, 1990). Morphologically, *Hanusia phi* is very similar to *Teleaulax acuta*, *Teleaulax merimbula* and the diplomorph of *Proteomonas sulcata* (Hill, 1991; Hill & Wetherbee,1986). However, phylogenetic trees constructed using nuclear 18S rRNA sequence from *Teleaulax* and *Proteomonas* (D. Hill & G. Saunders, unpublished data) suggest it would be wrong to assign *Hanusia phi* to either of these genera. For these reasons, we have proposed that a new genus,

Hanusia, be created to accommodate the species formerly known as *Cryptomonas* sp. Φ and that hereafter it be known as *Hanusia phi*.

Comparison of nucleomorph 18S rRNA genes (Fig. 12) clearly demonstrates that the organism studied by Douglas *et al*. (1991) was in fact *G. theta* rather than *Hanusia phi*. The three mismatches (Fig. 12) between the *G. theta* sequence and the sequence of Douglas *et al*. (1991) are most likely due to sequencing or *Taq* polymerase errors. *G. theta* was most probably used instead of *Hanusia phi* in other molecular studies such as those by Douglas (1988, 1991), Douglas & Dunford (1989, 1990*a*, *b*), Douglas & Turner (1991), Douglas *et al*. (1990, 1991), Wang & Liu (1991), McFadden *et al*. (1994*b*), Rensing *et al*. (1994) and Reith & Douglas (1990). Similarly, McFadden *et al*. (1994*a*) used the wrong culture for the *in situ* hybridization and karyotyping components of their study. It would appear that the cultures were switched at some stage after having left the Bigelow culture collection since the source cultures held there still match the original reports (Gillot & Gibbs, 1983; Hill & Wetherbee 1990). One can easily imagine a possible source of the mix-up when recalling that *G. theta* was originally known as *Cryptomonas* sp. θ (McKerracher & Gibbs, 1982). Indeed, the simple act of tilting the culture bottle on its side would convert θ to Φ. Whatever the case may be, this study serves to exemplify the importance of holding source cultures in carefully managed collections. It also serves as a reminder to avoid easily confused strain designations such as Φ and θ.

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