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Character evolution in heterotrophic euglenids

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This article attempts to describe the key morphological innovations associated with the evolutionary transitions between bacteriotrophy, eukaryotrophy, phototrophy, and osmotrophy in euglenids. Attention was focused on heterotrophic euglenids in an effort to establish a robust phylogenetic hypothesis for the group as a whole. We present a cladistic analysis of a large morphological data set from the following taxa: *Petalomonas*, *Entosiphon*, *Lentomonas*, *Ploeotia*, *Dinema*, *Distigma*, *Rhabdomonas*, *Menoidium*, *Peranema*, *Urceolus*, *Eutreptia*, and *Euglena*. The majority of the 37 characters and 97 states recognized were associated with the pellicle, the feeding apparatus, and the flagellar apparatus. In addition to having pellicle strips, *Petalomonas cantuscygni* possessed mitochondrial inclusions that were strikingly similar to the kinetoplasts found in kinetoplastids. *Dinema sulcatum* held a pivotal position in the phylogenetic tree and possessed many characters that bridged bacteriotrophic taxa with eukaryotrophic taxa. Distigmids and rhabdomonads formed a clade of osmotrophs that descended from eukaryotrophic ancestors, while *Urceolus cyclostomus* possessed a feeding apparatus, a putative photoreception apparatus and cytoskeletal features that clearly linked the phototrophs to eukaryotrophic ancestors. Evolutionary implications that emerged from these results were discussed.

Key words: Cladistics, Euglenozoa, Evolution, Morphology, Phylogeny.

Introduction

The Euglenida consists mostly of free-living flagellates with very diverse modes of nutrition. A number of heterotrophic euglenids (e.g., *Petalomonas* and *Ploeotia*) are limited to bacteriotrophy (Fig. 1a–c) while others (e.g., *Dinema* and *Peranema*) are capable of ingesting eukaryotic prey (Fig. 1f). We use the label "eukaryotroph" to distinguish the latter taxa from the bacteriotrophs (Triemer and Farmer 1991a; Leander and Farmer 2001b). Nonetheless, the most obvious euglenids in field samples tend to be phototrophs (e.g., *Euglena* and

Phacus) (Fig. 1g) that have acquired plastids via secondary endosymbiosis (Gibbs 1978, 1981). Other euglenids lack both plastids and the ability to phagocytize and are assumed to acquire their nutrients by absorbing organic molecules from the environment; collectively, these euglenids have been referred to as "osmotrophs".

Some osmotrophs (e.g., *Astasia longa* and *Hyalophacus*) are clearly derived from a photosynthetic ancestor by the loss of plastids (Gockel and Hachtel 2000), an event that occurred multiple times (Linton et al. 2000; Müllner et al. 2001). By contrast, the phylogenetic position(s) of os-

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motrophs like distigmids and rhabdomonads (Fig. 1d–e) is less obvious; however, small subunit (18S) rDNA sequences indicate that they diverged prior to the eukaryotrophic *Peranema* and the phototrophs (Preisfeld et al. 2000, 2001; Leander and Farmer 2001b; Müllner et al. 2001). We are interested in identifying the key morphological innovations in euglenid evolution associated with the transitions between bacteriotrophy, eukaryotrophy, phototrophy, and osmotrophy, but it is first necessary to establish a robust phylogenetic hypothesis for euglenids as a whole.

Much speculation about the evolutionary history of euglenids has occurred in the last four decades. Figure 2 illustrates the progression of thought regarding the phylogenetic relationships of taxa possessing the four primary modes of nutrition. When the distinction was not made in the literature, "phagotroph" refers to bacteriotrophs and eukaryotrophs collectively; we refer only to distigmids and rhabdomonads *sensu lato* (Leedale and Hibberd 1974; Cann 1986; Preisfeld et al. 2001) (Fig. 1d–e) as "osmotrophs" and not to those taxa that are clearly derived from phototrophs.

Prior to the widespread acceptance of the Serial Endosymbiotic Theory (SET), Leedale (1967) posited that the ancestral euglenid was a phototroph similar to the biflagellates *Eutreptia* and *Eutreptiella* and that osmotrophic and most bacteriotrophic euglenids shared a heterotrophic common ancestor that was similar to the colorless *Distigma* (Fig. 2a). However, the bacteriotroph *Entosiphon* was considered more closely related to eukaryotrophic euglenids. Leedale left the relationships between extant phototrophs, eukaryotrophs, and the group consisting of osmotrophs and bacteriotrophs unresolved (Fig. 2a).

During the following decade, the SET gained acceptance, and Leedale (1978) revised his scheme by suggesting that the ancestral euglenid was an osmotroph similar to *Distigma*, and the subsequent origin of phagotrophy united the bacteriotrophs, eukaryotrophs, and phototrophs (Fig. 2b). Leedale (1978) also accounted for the possibility that the phototrophs were derived from a phagotrophic ancestor that acquired plastids via endosymbiosis.

In the 1980's and early 1990's, the close relationship between bodonid kinetoplastids and euglenids became very clear, and many authors suggested that the ancestral euglenid was a phagotroph (Willey et al. 1988) (Fig. 2c). Willey and Wibel (1985b) reasoned that bacteriotrophic eu-

glenids with a simple feeding pocket, like *Petalomonas*, were more closely related to phototrophs than to eukaryotrophs and *Entosiphon*, which have a complex feeding apparatus comprised of rods and vanes (Fig. 2c). Dawson and Walne (1994) modified the argument by emphasizing the putative phylogenetic position(s) of osmotrophs. These authors suggested graphically that (1) osmotrophs and phototrophs are united by the loss of phagotrophy yet (2) plastids were subsequently gained in the phototrophic lineage (Fig. 2c). However, they contradictorily supported the notion that euglenid plastids originated from a phagotrophic ancestor (Dawson and Walne 1994).

Historically, phylogenetic hypotheses of euglenids have been based on intuition and an emphasis on a few morphological features. During the last decade, however, a great deal of information has accumulated for heterotrophic euglenids (Triemer 1986, 1988; Triemer and Fritz 1987, 1988; Farmer and Triemer 1988a, 1988b, 1994; Triemer and Farmer 1991a; 1991b; Belhadri and Brugerolle 1992; Belhadri et al. 1992; Linton and Triemer 1999, 2001; Leander and Farmer 2001a) that permits us to cladistically analyze their phylogenetic relationships in the context of a large ultrastructural data set. In this vein, we report new data on the ultrastructure of two *Petalomonas* taxa, *Dinema sulcatum*, *Distigma proteus*, *Menoidium cultellus*, *Rhabdomonas incurva* and *Urceolus cyclostomus*. Many morphological characters associated with the euglenid pellicle were identified in previous studies that evaluated the relationships among phototrophic euglenids (Leander and Farmer 2000a, 2000b, 2001a, 2001b). In this article, we expand the data matrix by identifying a number of characters and states associated with the flagellar and feeding apparatus. Our phylogenetic analyses support the hypothesis illustrated in Figure 2d, which clarifies many ambiguities present in the hypotheses outlined previously. Brief introductions to the three principal sources of morphological variation within heterotrophic euglenids follow.

Euglenid pellicle

The cytoskeleton or "pellicle" of euglenids consists of four main components: (1) the plasma membrane, (2) proteinaceous strips, (3) microtubules, and (4) tubular cisternae of endoplasmic reticulum (Leander and Farmer 2000a, 2001a). The morphological diversity found in the strips makes

them the most significant components from the perspectives of phylogeny and taxonomy. They are positioned immediately below the plasma membrane, run in parallel along the anteroposterior axis of the cell, articulate along their lateral borders, and may be arranged either helically or longitudinally (Triemer and Farmer 1991b; Leander and Farmer 2000a, 2001a). At the anterior end of many taxa, the strips line the canal and eventually terminate near the reservoir (Leander and Farmer 2000a). Strips may differ between taxa in how they are organized around the surface of the cell and in substructural details (Leander and Farmer 2000a, 2000b, 2001a, 2001b). Future characterization of differences in the organization of pellicular microtubules may also prove informative.

Flagellar apparatus

The basic organization and structure of the flagellar apparatus in euglenids is very similar to that of bodonids and diplonemids (Simpson 1997). There are usually two functional basal bodies designated "dorsal" and "ventral" that are associated with three flagellar roots (Surek and Melkonian 1986). The "intermediate" and "ventral" roots are associated with the ventral basal body, the dorsal root is associated with the dorsal basal body. A "striated fiber" (syn. basal body connective, Solomon et al. 1987) usually extends between the two basal bodies (Triemer and Farmer 1991b). The microtubules of the intermediate and dorsal roots are linked to bands of microtubules that support the canal and pellicle (syn. "dorsal pellicular microtubule band" – Surek and Melkonian 1986). The two flagella are designated as either "dorsal" (syn. anterior) or "ventral" (syn. posterior or recurrent) depending on the basal body from which they stem and are usually each supported by paraxial rods that differ slightly in structure (Dawson and Walne 1994; Simpson 1997). The flagellar apparatus may differ between taxa in the relative lengths of the flagella, in details of the flagellar transition zone, and in flagellar beat pattern. In addition, some taxa possess a photosensory "paraflagellar swelling" at the base of the dorsal flagellum (Kuźnicki et al. 1990).

Feeding apparatus

With respect to euglenozoans, eukaryotrophy occurs only within the Euglenida and is facilitated, in part, by a specialized feeding apparatus of "rods" and "vanes" (Triemer and Farmer 1991b). A feeding apparatus of this kind is also found in a number of bacteriotrophic euglenids (i.e., *Entosiphon* and *Ploeotia*) and a single rod-like structure (syn. nemadesm) is present in some bodonids (Hitchen 1974; Burzell 1975; Eyden 1977). The rods of euglenids are supported by amorphous material and microtubules that extend posteriorly from a discrete cytostome positioned near the canal opening (Triemer and Farmer 1991b). The lumen of the feeding apparatus and canal opening may merge into a common space called the "vestibulum", but an anterior "comb" or "cap" usually covers the opening of the cytostome. When the feeding apparatus protrudes from the vestibulum during feeding, the anterior cap moves to one side of the cytostome and consequently closes off the flagellar pocket (Nisbet 1974; Triemer and Fritz 1987). In most phagotrophic euglenids, the rods are closely associated with a "diaphragm-like" set of plicate folds or vanes that surround the cytostome (Triemer and Farmer 1991a, 1991b). As prey is engulfed, the vanes rotate open in a pinwheel-like fashion (Triemer and Fritz 1987).

All bodonids (e.g., *Bodo caudatus*) and some bacteriotrophic euglenids (e.g., *Petalomonas*) lack rods and vanes altogether and phagocytize with a simple pocket lined on one side with microtubules, an apparatus called the "MTR" (Brooker 1971; Brugerolle et al. 1979; Triemer and Farmer 1991a). MTR-like structures have been found in a variety of phototrophic euglenids, which implies that they are derived from bacteriotrophic ancestors (Willey and Wibel 1985a, 1985b; Surek and Melkonian 1986; Owens et al. 1988). Current evidence suggests that the MTR is not homologous to the more complex feeding apparatus of rods and vanes as both kinds of apparatus can occur within the same cell (e.g., *Dinema*) (Farmer and Triemer 1988a; Triemer and Ott 1990; Triemer and Farmer 1991a); in these cases, the MTR is assumed to be vestigial or to function in pinocytosis as it does in trypanosomatids (Preston 1969; Brooker 1971; Lom et al. 1980). Thus, there appear to be two unrelated types of feeding apparatuses: the MTR and the rod-and-vane-based apparatus.

The feeding apparatus may differ between taxa in the number of rods (e.g., 0, 2 or 3), the relative number of microtubules in the rods, the length of the rods relative to the length of the cell, the number of vanes (e.g., 4 or 5), and the presence of a phagocytic MTR.

Material and methods

Study organisms and culture conditions

The 21 taxa examined in this study, their locations either in the field or in culture collections, and the references used to score specific character states are listed in Table 1. *Petalomonas cantuscygni* was grown in an ASWP (Artificial Seawater for Protozoa) medium (http://www.ife.ac.uk/ccap/mediarecipes.html#aswp). *Distigma proteus, Rhabdomonas incurva, R. costata*, and *Menoidium cultellus* were grown in soil/water medium enriched with crushed barley (1 grain/200 ml) (Starr and Zeikus 1993). *Peranema trichophorum* was grown in a *Peranema* medium (PER, The Culture Collection of Algae and Protozoa, Ambleside, Cumbria, UK) containing "Complan" (H. J. Heinz Co. Ltd., Hayes Park, Middlesex, UK). *Eutreptia pertyi, Euglena acus, E. laciniata, E. terricola* and *Lepocinclis buetschlii* were grown in a soil/water (Gr+/NH$_4$) medium enriched with calcium carbonate (0.1 g / 200 ml) and ammonium magnesium phosphate hexahydrate (0.1 g/200 ml) (Starr and Zeikus 1993). *Euglena mutabilis* was grown in equal parts of *Euglena* medium (EG, Greenblatt and Schiff

1959) and soil water extract. The cultured taxa were grown at 20 °C and 12:12 L:D cycle; all other taxa in the analysis were isolated from the field (Table 1).

Electron microscopy

Cells of *D. proteus, E. pertyi, E. acus, E. laciniata, E. mutabilis, E. terricola, L. buetschlii, M. cultellus, P. trichophorum, R. costata*, and *R. incurva* were prepared for scanning and transmission electron microscopy (SEM and TEM, respectively) using the protocols described by Leander and Farmer (2000a, 2001a). Cells of *D. sulcatum, E. sulcatum, L. applanatum, P. cantuscygni, P. mediocanellata, P. costata, P. vitrea*, and *U. cyclostomas* were prepared for SEM and TEM by the protocols described in Triemer (1985, 1986) and Farmer and Triemer (1988a). SEM data were presented on a black background using Adobe Photoshop 5.0.

Phylogenetic analysis

Ninety-seven character states within 37 morphological characters were recognized and scored for 19 euglenid taxa and two kinetoplastid taxa (Tables 2 and 3).

Table 1. The taxa examined in this study.

Taxon	Source[a]	References
Bodo saltans	–	Brooker 1971; Vickerman 1991
Bodo caudatus	–	Brooker 1971; Vickerman 1991
Petalomonas mediocanellata	Field	Farmer and Triemer 1988a; Triemer and Farmer 1991b
Petalomonas cantuscygni	CCAP 1259/1	Farmer and Triemer 1988a; Triemer and Farmer 1991b
Entosiphon sulcatum	Field	Triemer and Fritz 1987; Triemer and Farmer 1991b; Belhadri et al. 1992
Lentomonas applanatum	Field	Triemer and Farmer 1991b; Farmer and Triemer 1994
Ploeotia costata	Field	Triemer 1986; Farmer and Triemer 1988a; Triemer and Farmer 1991b; Linton and Triemer 1999, 2001
Ploeotia vitrea	Field	Farmer and Triemer 1988a; Triemer and Farmer 1991b
Rhabdomonas costata	UTEX LB 1278	Leedale and Hibberd 1974
Rhabdomonas incurva	UTEX LB 573	–
Menoidium cultellus	UTEX LB 528	Leedale and Hibberd 1974 (*M. bibacillatum*)
Distigma proteus	UTEX LB 508	Leander and Farmer 2000a, 2001b
Dinema sulcatum	Field	Triemer and Farmer 1991b
Peranema trichophorum	CBSC WW-13-1838	Nisbet 1974; Hilenski and Walne 1885a; Triemer and Farmer 1991b; Belhadri et al. 1992; Leander and Farmer 2001b
Urceolus cyclostomus	Field	Triemer and Farmer 1991b
Eutreptia pertyi	UTEX 1290	Leander and Farmer (unpublished)
Euglena mutabilis	SAG 1224-9a	Leander and Farmer 2000a
Euglena laciniata	UTEX LB 1312	Leander and Farmer (unpublished)
Euglena terricola	UTEX LB 1310	Leander and Farmer (unpublished)
Euglena acus	UTEX LB 1316	Leander and Farmer 2001b
Lepocinclis buetschlii	UTEX LB 523	Leander and Farmer (unpublished)

[a] CBSC, Carolina Biological Supply Company, Burlington, NC; CCAP, Culture Collection of Algae and Protozoa, Cumbria, U.K.; SAG, Sammlung von Algenkulturen Göttingen; UTEX, Culture Collection of Algae at the University of Texas, Austin, TX.

Table 2. Thirty-seven morphological characters and 97 states associated with the pellicle, feeding apparatus, and flagellar apparatus of euglenids.

Character	Character States
1) Strips	0, absent; 1, present
2) Orientation of strips	0, longitudinal; 1, helical
3) Strips lining the canal	0, absent; 1, present
4) P[a]	0, 5–8; 1, 10–12; 2, 14; 3, 18–20; 4, 28–32; 5, ≥38
5) W_A[b]	0, 0; 1, 1
6) Posterior strip reduction	0, absent; 1, present
7) Pattern of posterior strip reduction	0, partial[c]; 1, linear/pseudolinear; 2, exponential
8) W_P[d]	0, 1; 1, 2; 2, 3
9) Binary pattern of pores	0, absent; 1, present
10) Cell plasticity	0, rigid; 1, plastic
11) Transverse shape of cell	0, ovoid; 1, circular; 2, pentagonal; 3, flat
12) Frames	0, thin; 1, robust
13) Overhang	0, absent; 1, present
14) Prearticular projections	0, absent; 1, delicate; 2, thread-like; 3, tooth-like
15) Postarticular projections	0, absent; 1, indented plate
16) Doublet identity	0, absent; 1, heels, 2, arches; 3, trough & flat
17) Arch width : heel width	0, indistinguishable; 1, arches ≈ 3X heel[e]; 2, arches ≥ 15X heel; 3, arches ≈ heels; 4, arches ≈ 5X heels; 5, heel ≥ 3X arches
18) Articulation zones bifurcate[f]	0, absent; 1, present
19) Flagellar strip	0, absent; 1, present
20) Fused frames w/discontinuities	0, absent; 1, present
21) Supporting elements around canal	0, absent; 1, present
22) Length of ventral flagellum	0, ≥ 2X cell length; 1, < 2X cell length
23) Ventral flagellum emergent	0, present; 1, absent
24) Dorsal flagellar beat pattern	0, whiplike/power-recovery stroke; 1, held straight with twitching at tip; 2, highly mobile; 3, figure eight/lasso
25) Paraflagellar swelling	0, absent; 1, present
26) Flagellar transition zone	0, internal elements; 1, hollow
27) Phagotrophic MTR	0, present; 1, absent
28) Anterior feeding cap/comb	0, absent; 1, present
29) Feeding apparatus: rods	0, absent; 1, 2; 2, 3
30) Feeding apparatus: vanes	0, absent; 1, 5 vanes - plicate; 2, 4 vanes
31) Rel. # of microtubules in rods	0, few; 1, many
32) Rod length : cell length	0, 1:1; 1, ≤0.5:1
33) Mode of nutrition	0, bacteriotroph; 1, eukaryotrophy; 2, osmotrophy; 3, phototrophy
34) Plastid morphology	0, shield; 1, stellate; 2, discoidal
35) Large paramylon grains	0, absent; 1, present
36) Mitochondrial inclusion bodies	0, present; 1, absent
37) Cell length [g]	0, < 20 µm; 1, ≥ 20 µm

[a] "P" refers to the maximum number of strips around the cell periphery (Leander and Farmer 2000a).

[b] "W_A" refers to the number of anterior whorls of exponential strip reduction (Leander and Farmer 2000a).

[c] Strips at the posterior end converge on a common line; most of the strips (e.g., 41 of 50) terminate on one side of the line and show an alternate pattern of strip reduction (Leander and Farmer 2002).

[d] "W_P" refers to the number of posterior whorls of exponential strip reduction excluding primed Roman numerals (Leander and Farmer 2000a, 2000b).

[e] In *Dinema*, scored from the six strips near the flagellar strip.

[f] "Bifurcate" refers to a raised articulation zone bordered by a pronounced keel and overhang (e.g., Farmer and Triemer 1994).

[g] Scored from relaxed cells.

The matrix of unordered character states was analyzed parsimoniously using the branch-and-bound algorithm and the default settings in PAUP* 4.0 (Swofford 1999). Two bodonids, *Bodo saltans* and *B. caudatus*, were designated as the outgroup based on previous studies on the molecular phylogeny and comparative morphology of euglenozoans (Willey et al. 1988; Triemer and Farmer 1991a, 1991b; Montegut-Felkner and Triemer 1997; Linton et al. 2000; Preisfeld et al. 2000, 2001). The support of the data for each node on the most parsimonious tree(s) was estimated using decay indices using Autodecay 4.0.2 (Eriksson 1998) and nonparametric bootstrap percentages (Felsenstein 1985) from 500 replications using PAUP* 4.0. MacClade 3.03 was used to phylogenetically map character state changes.

Results

Comparative morphology of heterotrophic euglenids

Tables 2 and 3 summarize the character states present in the taxa examined in this study. The descriptions in this section will be restricted to select-

ed ultrastructural features of *P. mediocanellata*, *P. cantuscygni*, *D. sulcatum*, *P. trichophorum*, *R. incurva*, *D. proteus*, and *U. cyclostomus*. Ultrastructural features of *Entosiphon*, *Ploeotia*, *Lentomonas*, and the phototrophic taxa have been described in depth elsewhere (Triemer 1986, 1988; Triemer and Fritz 1987, 1988; Farmer and Triemer 1988a, 1988b, 1994; Triemer and Farmer 1991a, 1991b; Belhadri and Brugerolle 1992; Belhadri et al. 1992; Linton and Triemer 1999, 2001; Leander and Farmer 2000a, 2001a, 2001b).

Both *P. mediocanellata* and *P. cantuscygni* are small bacteriotrophs possessing a dorsal flagellum that beats by rapidly twitching the distal end (Cann and Pennick 1986). *Petalomonas mediocanellata* possessed a very short ventral flagellum that emerged from the vestibulum (Fig. 1a), whereas *P. cantuscygni* lacked one entirely (Fig. 1b). The pellicle of *P. cantuscygni* consisted of eight longitudinal strips that were more or less fused at the articulation zones (Fig. 1b and 3); however, *P. mediocanellata* appeared to lack a pellicle of proteinaceous strips altogether (Fig. 1a; Farmer and Triemer 1988a). Both

Table 3. Matrix of 37 morphological characters for 21 taxa used in the parsimony analysis to infer phylogenetic relationships. All characters are unordered and equal in weight. The symbols for the characters and character states are defined in Table 2. N = unknown and dashes denote inapplicable characters.

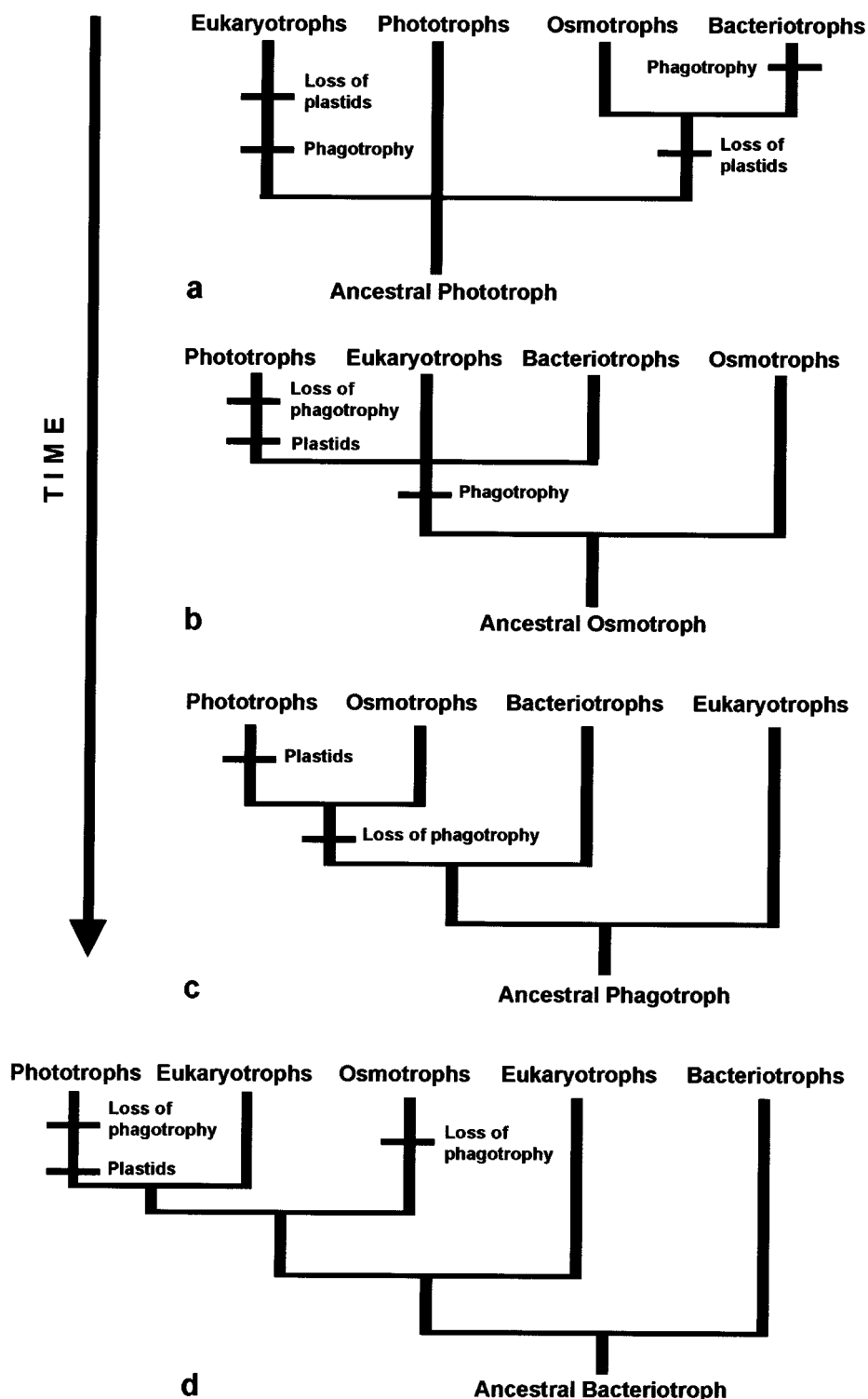| Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
Bodo saltans	0	–	0	–	–	–	–	–	0	0	0	–	–	–	–	–	–	–	0	0	0	0	0	0	0	0	0	0	0	0	–	–	0	–	0	0	0
Bodo caudatus	0	–	0	–	–	–	–	–	0	0	0	–	–	–	–	–	–	–	0	0	0	0	0	0	0	0	0	0	0	0	–	–	0	–	0	0	0
Petalomonas mediocanellata	0	–	0	–	–	0	–	–	0	0	0	–	–	–	–	–	–	–	0	0	0	1	0	1	0	0	0	0	0	0	–	–	0	–	0	0	0
Petalomonas cantuscygni	1	0	0	0	–	0	–	–	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	–	–	0	–	0	0	0
Entosiphon sulcatum	1	0	0	1	–	0	–	–	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	1	2	2	1	0	0	–	0	1	0
Lentomonas applanatum	1	0	0	1	–	0	–	–	0	0	0	1	0	0	0	2	1	0	0	0	0	0	0	1	0	0	1	1	1	2	0	0	0	–	0	1	0
Ploeotia costata	1	0	0	1	–	0	–	–	0	0	2	0	1	0	0	2	2	1	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	–	0	1	0
Ploeotia vitrea	1	0	0	1	–	0	–	–	0	0	0	1	0	0	0	2	1	1	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	–	0	1	0
Rhabdomonas costata	1	0	0	2	–	0	–	–	0	0	1	0	–	0	0	3	–	–	0	1	1	1	1	2	0	1	1	–	0	0	–	–	2	–	1	1	1
Rhabdomonas incurva	1	0	0	2	–	0	–	–	0	0	1	0	–	0	0	3	–	–	0	1	1	1	1	2	0	1	1	–	0	0	–	–	2	–	1	1	0
Menoidium cultellus	1	0	0	3	–	0	–	–	0	0	3	0	–	0	0	0	–	–	0	1	1	1	1	2	0	1	1	–	0	0	–	–	2	–	1	1	1
Distigma proteus	1	1	0	3	–	0	–	–	0	1	1	0	1	0	0	0	3	0	0	0	1	1	0	2	0	1	1	–	0	0	–	–	2	–	1	1	1
Dinema sulcatum	1	1	0	3	–	0	–	–	0	1	1	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	1	1	1	2	1	0	1	–	1	1	0
Peranema trichophorum	1	1	1	5	0	1	0	–	0	1	1	0	1	0	0	0	3	0	1	0	1	1	0	1	0	1	1	1	1	2	1	1	1	–	1	1	1
Urceolus cyclostomus	1	1	1	5	0	1	N	N	0	1	1	0	1	0	0	0	5	0	0	0	0	1	1	1	1	1	1	0	1	2	1	1	1	–	1	1	1
Eutreptia pertyi	1	1	1	5	0	1	1	0	0	1	1	0	1	0	0	0	3	0	0	0	0	1	0	2	1	1	1	–	0	0	–	–	3	0	1	1	1
Euglena mutabilis	1	1	1	5	0	1	1	1	0	1	1	0	1	1	1	0	3	0	0	0	0	1	1	–	1	1	1	–	0	0	–	–	3	0	1	1	1
Euglena laciniata	1	1	1	5	1	1	2	1	1	1	1	0	1	2	1	0	3	0	0	0	0	1	1	3	1	1	1	–	0	0	–	–	3	1	1	1	1
Euglena terricola	1	1	1	5	1	1	2	1	1	1	1	0	1	2	1	0	3	0	0	0	0	1	1	3	1	1	1	–	0	0	–	–	3	1	1	1	1
Euglena acus	1	1	1	4	1	1	2	1	0	1	1	1	1	3	1	0	4	0	0	0	0	1	1	3	1	1	1	–	0	0	–	–	3	2	1	1	1
Lepocinclis buetschlii	1	1	1	4	1	1	2	1	0	1	1	1	1	3	1	0	4	0	0	0	0	1	1	3	1	1	1	–	0	0	–	–	3	2	1	1	1
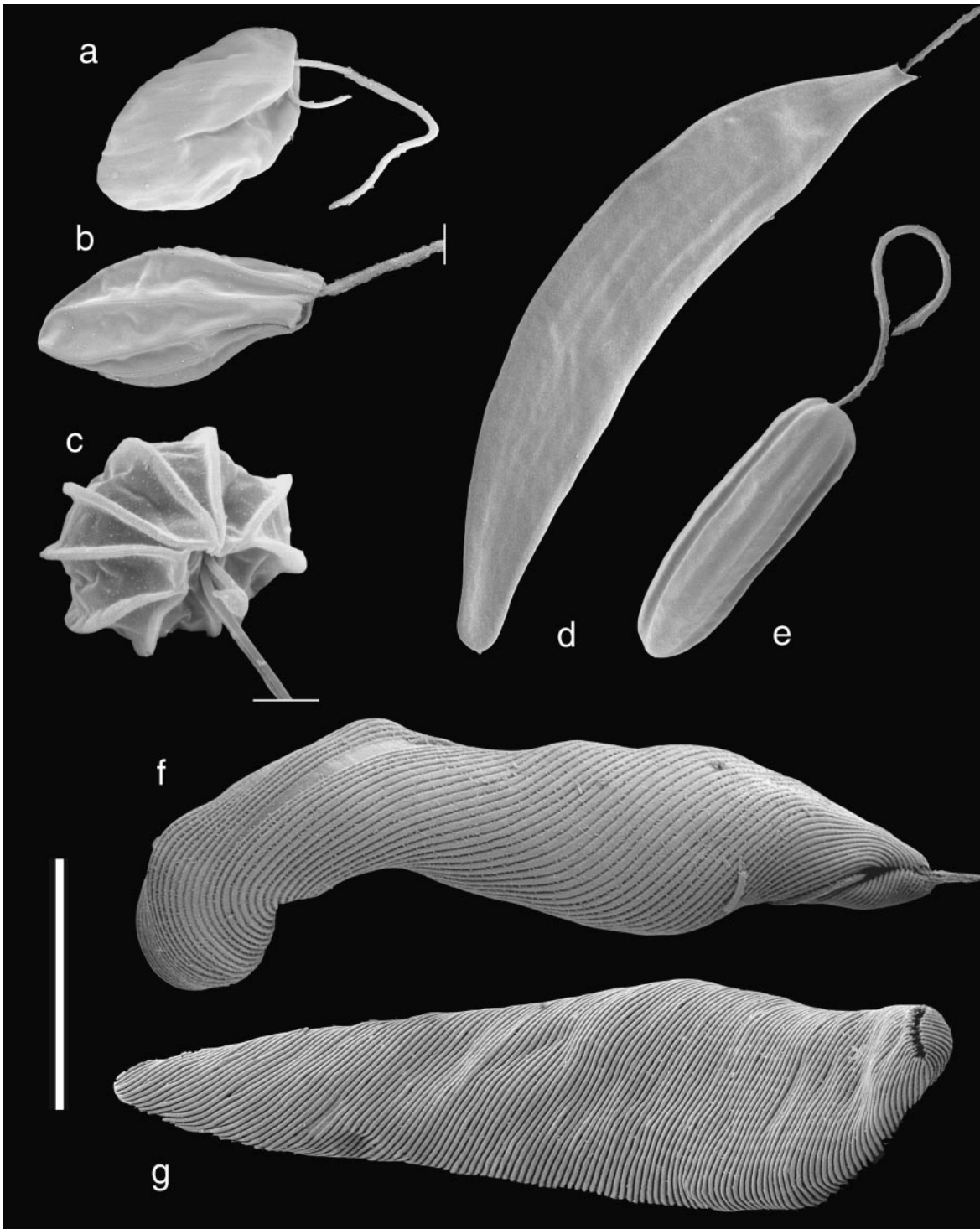
Fig. 1. Scanning electron micrographs (SEM) of some of the investigated taxa showing diversity in pellicle morphology and cell size (Bar = 10 μm). **a.** *Petalomonas mediocanellata* (bacteriotroph). **b.** *Petalomonas cantuscygni* (bacteriotroph). **c.** *Ploeotia vitrea* (bacteriotroph). **d.** *Menoidium cultellus* (osmotroph). **e.** *Rhabdomonas incurva* (osmotroph). **f.** *Peranema trichophorum* (eukaryotroph). **g.** *Euglena mutabilis* (phototroph).

P. mediocanellata and *P. cantuscygni* possessed mitochondrial inclusion bodies that were similar in appearance to kinetoplasts; the inclusions had an ordered nature to them and were centrally located in a portion of the mitochondrion that lacked cristae (Fig. 3 and 4). Like kinetoplasts, these inclusion bodies were found only in mitochondrial profiles positioned near the flagellar basal bodies.

Dinema sulcatum, P. trichophorum, and *U. cyclostomus* are eukaryotrophs possessing plastic

TIME

a — Eukaryotrophs Phototrophs Osmotrophs Bacteriotrophs; Loss of plastids; Phagotrophy; Phagotrophy; Loss of plastids; Ancestral Phototroph

b — Phototrophs Eukaryotrophs Bacteriotrophs Osmotrophs; Loss of phagotrophy; Plastids; Phagotrophy; Ancestral Osmotroph

c — Phototrophs Osmotrophs Bacteriotrophs Eukaryotrophs; Plastids; Loss of phagotrophy; Ancestral Phagotroph

d — Phototrophs Eukaryotrophs Osmotrophs Eukaryotrophs Bacteriotrophs; Loss of phagotrophy; Plastids; Loss of phagotrophy; Ancestral Bacteriotroph

pellicles and crystalline paramylon grains. *Dinema sulcatum* and *P. trichophorum* possessed two emergent flagella with the ventral flagellum positioned tightly within a specialized pellicular strip designated as the "flagellar strip" (Fig. 5–8). The flagellar strip of *P. trichophorum* is unique in possessing a significantly broader heel that accommodated the ventral flagellum (Roth 1959; Hilenski and Walne 1985a)(Fig. 7–8). In *D. sulcatum*, the flagellar strip was very broad but otherwise similar in morphology to the majority of the strips, particularly those positioned to the right of the flagellar strip (as viewed from the posterior end, Fig. 5–6). In transverse view the flagellar strip appeared to be closely associated with the superficial supporting rod of the feeding apparatus (Fig. 6). The six to seven strips to the left of the flagellar strip (as viewed from the posterior end) possessed a substructure that was more common to euglenids in that the frames were sigmoidal with distinct arches and heels (Fig. 6). The arches were rounded and approximately 3-times the width of the heels. The number of strips around the cell periphery in *D. sulcatum* ranged from 18–22 (n = 15).

The morphology of the canal region in euglenids is diverse. In *D. sulcatum*, strips surrounded the canal opening but did not extend into the pocket. The anterior portion of the flagellar opening consisted of a vestibulum into which both the flagellar and feeding pockets opened. The flagellar reservoir only formed a complete enclosure well into the cell (Fig. 6). *Peranema trichophorum* possessed a distinct canal that was internally lined by strips for some of its length (Nisbet 1974). The canal was also surrounded by supporting elements of unknown composition that did not form a complete ring around the canal (Fig. 9). Split-ringed elements surrounding the canal (syn. scrolls) have also been observed in *Rhabdomonas* and *Menoidium* (Leedale and Hibberd 1974) (Fig. 10) and other rhabdomonads (Cann 1986). Similar structures were present around the canal of *D. proteus*. These "peri-canalar fibers" were opaque and slightly smaller in diameter than microtubules (Fig. 11). Although in *D. proteus* strips did not line the canal lumen, the canalar microtubules and the "peri-canalar fibers" served to delineate the canal from the reservoir.

The entire canal of *U. cyclostomus* was lined by pellicle strips and was not supported by split-ringed elements (Fig. 12). The number of strips lining the canal, C, was 38–42, which was equivalent to the number of strips surrounding the cell periphery, P; therefore, there was no distinct whorl of exponential strip reduction near the anterior end ($W_A = 0$). In addition to having a paraxial rod, the dorsal flagellum of *U. cyclostomus* had a localized, electron-opaque matrix associated with it. This paraflagellar swelling was positioned on the side of the axoneme away from the paraxial rod and in the anterior half of the reservoir (Fig. 13). A separate electron-dense material was associated with a band of sub-reservoir microtubules and positioned in the anterior portion of the reservoir corresponding to that of the paraflagellar swelling (Fig. 13). Neither this putative "stigma" nor the paraflagellar swelling was ever detected in the canal.

Fig. 2. Competing hypotheses for the phylogenetic relationships of eukaryotrophic (e.g., *Dinema* and *Peranema*), bacteriotrophic (e.g., *Petalomonas* and *Ploeotia*), osmotrophic (specifically distigmids and rhabdomonads), and phototrophic (e.g., *Euglena* and *Eutreptia*) euglenids. The vertical arrow indicates the relative time each hypothesis was proposed. **a.** Leedale (1967) suggested that the ancestral euglenid was a phototroph that subsequently gave rise to phagotrophs (eukaryotrophs and bacteriotrophs) and osmotrophs by the loss of plastids. **b.** Leedale (1978) suggested that the ancestral euglenid was an osmotroph (like *Distigma*) that subsequently gave rise to phagotrophs. Phototrophic euglenids gained plastids from a phagotrophic ancestor through secondary enodsymbiosis. **c.** Willey and Wibel (1985b) and Dawson and Walne (1994) proposed similar hypotheses suggesting that the ancestral euglenid was a phagotroph (whether it was bacteriotrophic or eukaryotrophic was unspecified). Willey and Wibel (1985b) used the absence of feeding rods (syn. nemadesms) to suggest that bacteriotrophs such as *Petalomonas* and *Calycimonas* were more closely related to phototrophs than to eukaryotrophs (e.g., *Peranema*). Dawson and Walne (1994) suggested that osmotrophs were descended directly from phagotrophs and indicated graphically that these osmotrophs share a non-phagotrophic (presumably osmotrophic) ancestor with phototrophs. **d.** In contrast to the previous schemes, the hypothesis outlined in this paper proposes that the ancestral euglenid was a bacteriotroph similar in many ways to *Petalomonas*. Following the origin of eukaryotrophy, different eukaryotrophic lineages are thought to have independently led to osmotrophic and phototrophic modes of nutrition.
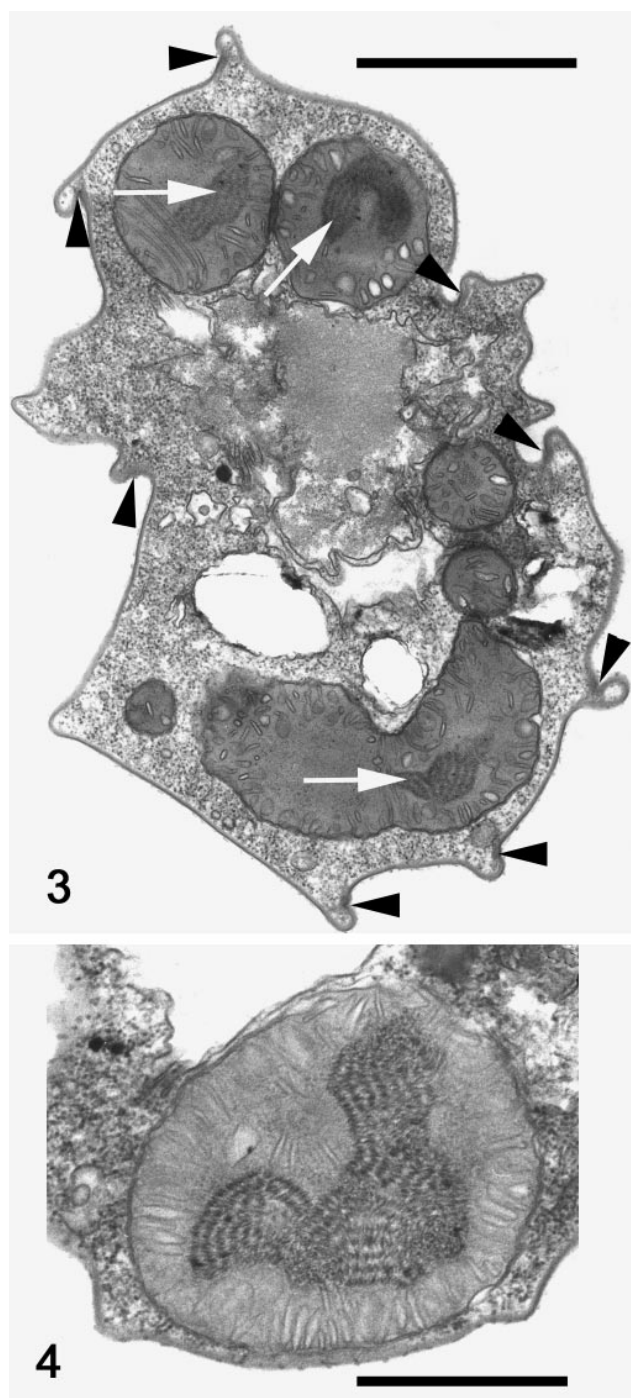
Fig. 3–4. Transmission electron micrographs (TEM) of *Petalomonas cantuscygni* showing mitochondrial inclusion bodies that resemble kinetoplasts. **3.** TEM near the anterior end of the cell showing multiple mitochondrial profiles possessing inclusion bodies (arrows) and pellicle strips. Arrowheads mark the articulation zones (Bar = 1 μm). **4.** A high magnification TEM showing the organized substructure of the inclusion bodies (Bar = 0.25 μm).

Phylogeny of heterotrophic euglenids

The branch-and-bound analysis produced a single most parsimonious tree (Fig. 14). *Petalomonas mediocanellata* was the earliest diverging taxon in our ingroup, and the remaining taxa fell within clade **B**, which represents the Euglenida (i.e., those taxa with pellicle strips). *Petalomonas cantuscygni* diverged before all other euglenids forming the sister taxon to clade **C**, which contained two major subclades of heterotrophic euglenids, namely clades **D** and **I**. Clade **D** consisted of four bacteriotrophic taxa possessing a feeding apparatus of rods and vanes and a resolved internal topology showing *E. sulcatum* as the sister taxon to a clade containing *Lentomonas* and *Ploeotia* (clade **E**, Fig. 14). Clade **I** consisted of four osmotrophic taxa and an internal topology showing the plastic *D. proteus* as the sister taxon to the rigid rhabdomonads (clade **J**). The phototrophic euglenids formed a monophyletic group (clade **N**) with an internal topology showing *Eutreptia* diverging before *Euglena*. The eukaryotrophs *P. trichophorum* and *U. cyclostomus* clustered with the phototrophs to form clade **L**, where *U. cyclostomus* was more closely related to the phototrophs than to *P. trichophorum* (Fig. 14). The eukaryotroph *D. sulcatum* held a pivotal position in the tree in that it bridged the obligate bacteriotrophs with clade **H**, which was comprised of the other eukaryotrophs, the osmotrophs, and the phototrophs. In general, the cladistic analysis indicated that bacteriotrophs and eukaryotrophs as separate groups are paraphyletic and that osmotrophs *sensu stricto* and phototrophs as separate groups are monophyletic (Fig. 14).

Discussion

"Petalomonads" and the origin of euglenids

The genus *Petalomonas* as currently defined is paraphyletic. The euglenozoan with the most plesiomorphic character states appears to be *P. mediocanellata*, which possesses two heterodynamic flagella, a mitochondrial inclusion near the flagellar bases, three asymmetrically arranged flagellar roots, and a phagocytic MTR. It is difficult to identify *P. mediocanellata* as either a bodonid or a euglenid, in part because it lacks distinct pellicle strips yet possesses the peculiar flagellar beat pattern of phagotrophic euglenids (the dorsal flagellum is held straight and the anterior tip twitches).

Nonetheless, this taxon has many of the characters that are common to both groups and appears to be an extant form that best represents the most recent common ancestor of bodonids and euglenids.

Like *P. mediocanellata*, the earliest diverging euglenid, *P. cantuscygni*, possesses mitochondrial inclusion bodies near the flagellar bases that are almost certainly homologous to kinetoplasts. The recognition of kDNA in these inclusion bodies

would, of course, eliminate all doubt. Nonetheless, from an evolutionary perspective, *P. cantuscygni* is a prediction come true. By possessing (1) most of the euglenozoan symplesiomorphies, (2) the best synapomorphy for the Euglenida (pellicle strips), and (3) the best synapomorphy for the Kinetoplastida (kinetoplasts), this extant taxon succinctly bridges a group containing intracellular parasites (e.g., trypanosomatids) with a group containing
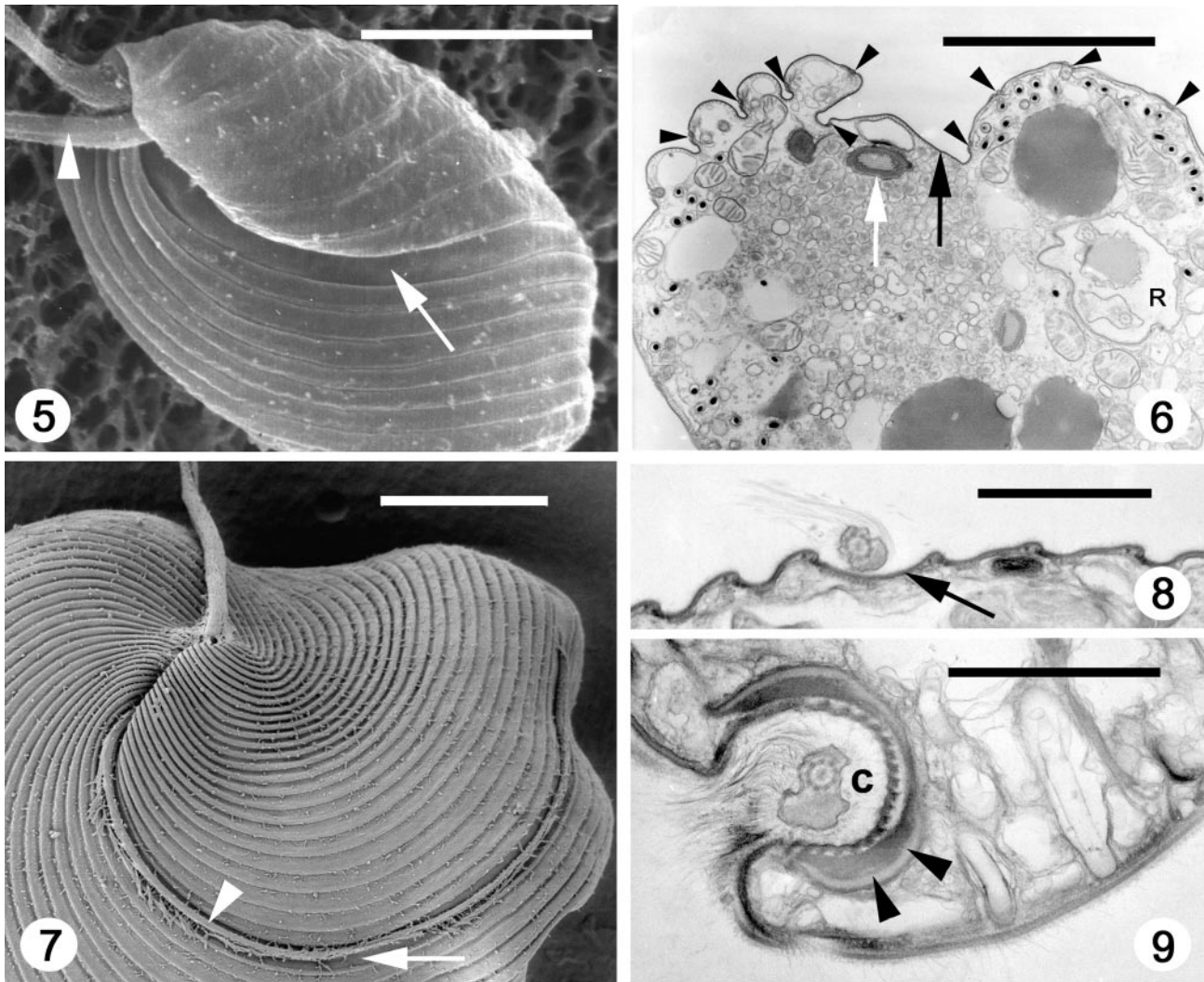
Fig. 5–9. The "flagellar strips" of *Dinema* and *Peranema*. **5.** SEM of *D. sulcatum* showing the flagellar strip (arrow) and the ventral flagellum (arrowhead) (Bar = 5 μm). **6.** TEM of *D. sulcatum* showing the flagellar strip (black arrow) and the different morphology of the adjacent strips. A superficial rod of the feeding apparatus (white arrow) is in close association with the flagellar strip. The articulation zones between the strips are marked with arrowheads. The strips to the left of the flagellar strip possess distinct heels and rounded arches; the strips to the right of the flagellar strip are flattened as described by Leander and Farmer (2001a). The reservoir (R) is also present (Bar = 3 μm). **7.** SEM of the anterior end of *P. trichophorum* showing the ventral flagellum (arrowhead) running along the flagellar strip (arrow) (Bar = 5 μm). **8.** TEM of *P. trichophorum* showing the broad heel of the flagellar strip (arrow) (Bar = 1 μm). **9.** TEM of *P. trichophorum* showing supporting elements (arrowheads) around the canal (C) (Bar = 1 μm).

free-living phototrophs (e.g., euglenophytes). However, this union of character states begs the question: Is *P. cantuscygni* more closely related to euglenids or kinetoplastids? Phylogenetic analyses of small subunit rDNA using non-euglenozoan outgroups incontrovertibly demonstrate that *P. cantuscygni* forms the sister taxon to the other eu-glenids in the ingroup (Müllner et al. 2001; Preis-feld et al. 2000, 2001). We can infer from these data that the Euglenida was derived from an ancestor possessing kinetoplasts and, consequently, the Kinetoplastida is paraphyletic. Therefore, from a taxonomic point of view, *P. cantuscygni* is rather problematic.
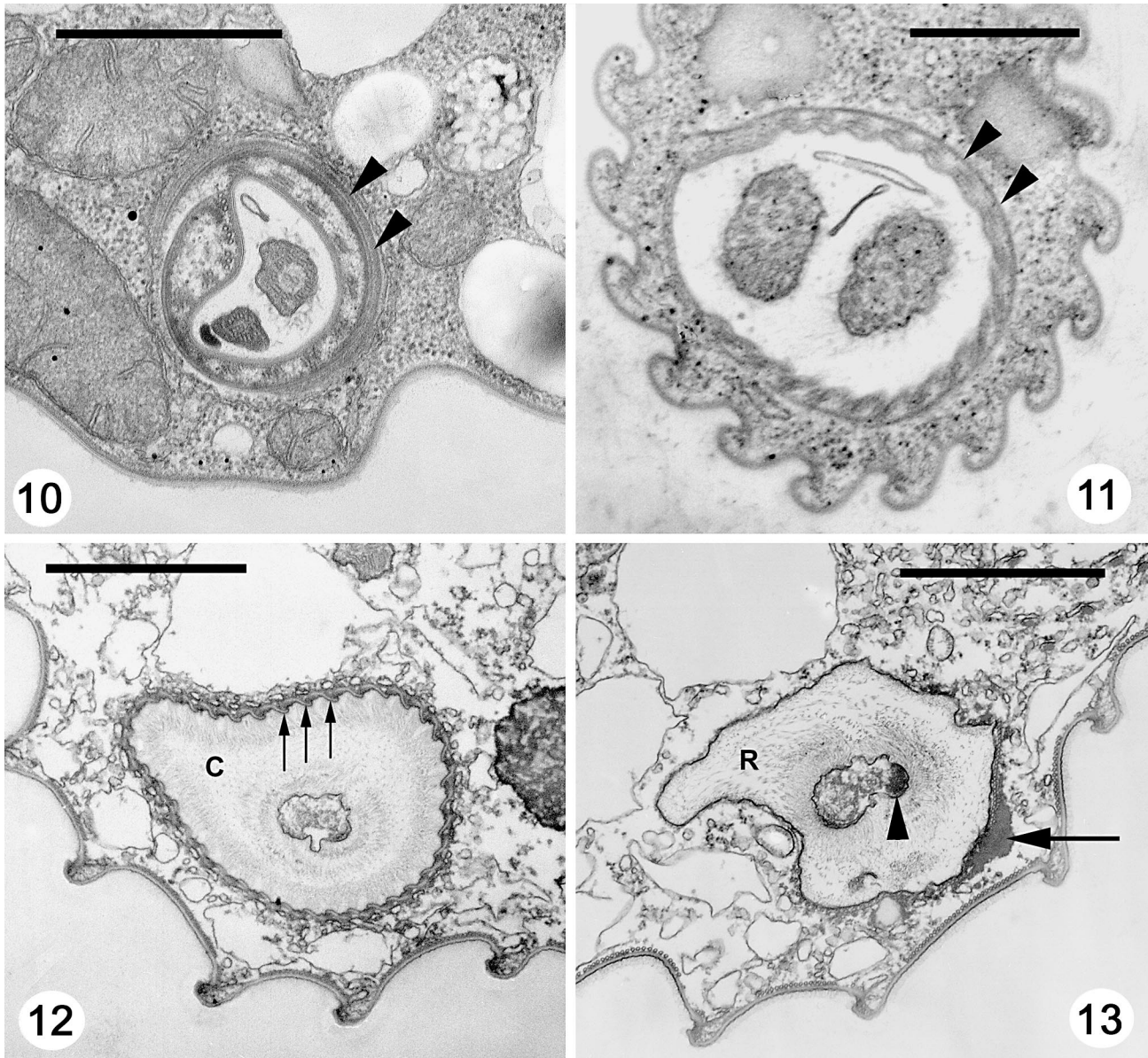
Fig. 10–13. Transverse TEMs comparing the canal morphology of *Rhabdomonas, Distigma,* and *Urceolus.* **10.** The canal of *R. costata* is surrounded by supporting elements called "scrolls" (arrowheads) (Leedale and Hibberd 1974) (Bar = 1 μm). **11.** The canal of *D. proteus* is also surrounded by supporting elements (arrowheads) (Bar = 0.5 μm). **12.** TEM of *U. cyclostomus* showing a canal (C) lined by the same number of strips (arrows) that surround the cell periphery, P; in this cell, P = C = 38. Supporting elements do not surround the canal (Bar = 1.5 μm). **13.** TEM at the level of the reservoir (R) of *U. cyclostomus* (eukaryotroph) showing a paraflagellar swelling (arrowhead) on the dorsal flagellum and a putative stigma (arrow) (Bar = 1.5 μm).

Ploeotiids and the origin of the feeding apparatus

Bodonids and petalomonads possess a relatively simple phagocytic MTR, which functions in bacteriotrophy. With the exception of petalomonads, all of the phagotrophic euglenids share a similar type of feeding apparatus consisting of rods and vanes. Rod-like structures have become associated with the MTR in some bodonids (Burzell 1975; Eyden 1977); however, as mentioned previously, these structures are suspected to be analogs of the supporting rods found in euglenids (Farmer and Triemer 1988a; Triemer and Ott 1990; Triemer and

Farmer 1991a). Because the rod-and-vane feeding apparatus is found in so many different taxa, the most parsimonious inference is that rods and vanes appeared fairly early in euglenid evolution; our analysis suggests that they evolved in the most recent common ancestor of clade C (Fig. 14–15). Our analysis also indicates that rods supported by relatively few microtubules is a derived state for clade E, and rods supported by many microtubules is homologous in *E. sulcatum* and the eukaryotrophs (Fig.14–15).

Two unanswered questions remain: (1) Did a rod-and-vane-based feeding apparatus evolve independently of a phagocytic MTR within eu-
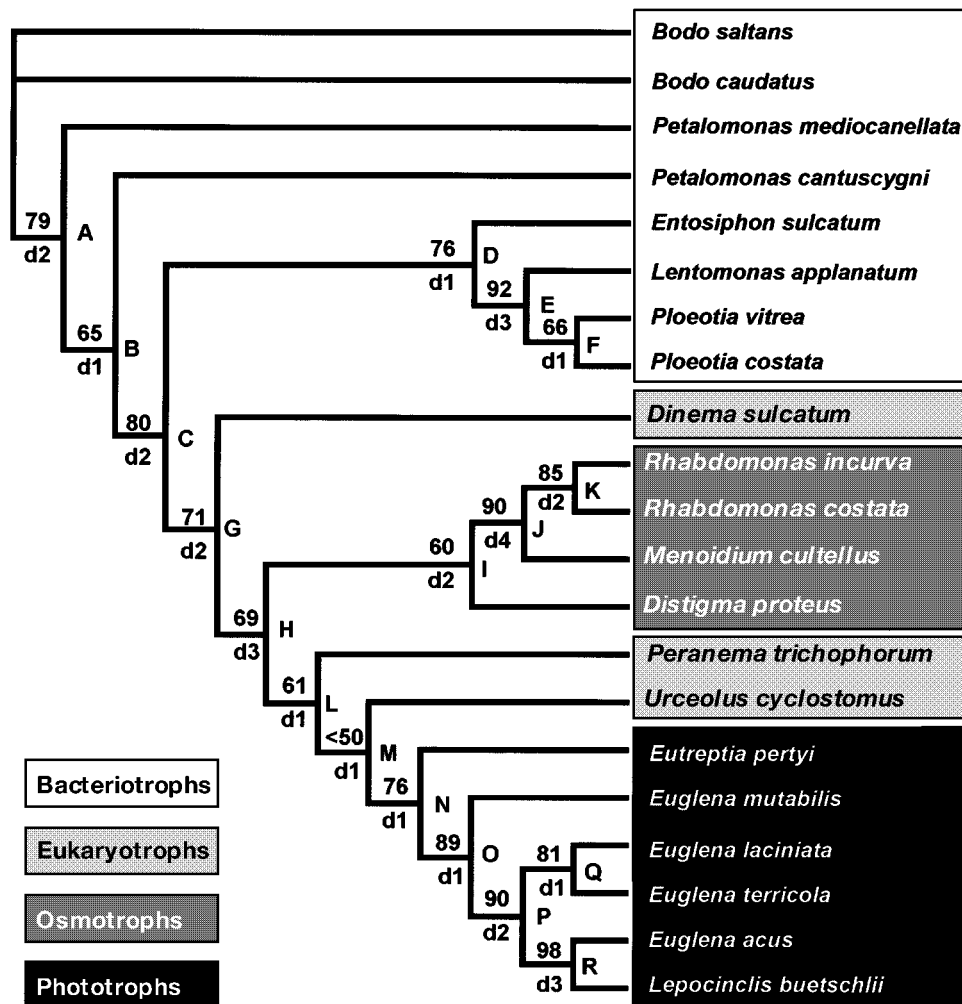

Fig. 14. The single most parsimonious tree using the branch-and-bound algorithm on a matrix of morphological character states from 21 euglenozoan taxa (Tables 2 and 3). All 37 characters (consisting of 97 states) were unordered and of equal weight. The numbers above the stems are bootstrap percentages from 500 replications; the symbols below each stem represent decay indices. Each clade may be referred to by the letter positioned near the respective node. The consistency index (excluding uninformative characters) was 0.79 and the retention index was 0.90. Character evolution in the nutritional mode of euglenids is indicated.

glenids and (2) if so, why? Both kinds of feeding apparatus are present in some euglenids (e.g., *Dinema*), where the MTR is assumed either to be vestigial or to function in pinocytosis rather than phagotrophy (Preston 1969; Lom et al. 1980). The presence of both kinds of apparatus in the same cell implies that the rod-and-vane based apparatus evolved independently from the MTR. There have been reports that in *Calycimonas physaloides* and *Petalomonas hovassei* four vanes exist in the absence of rods (Mignot 1966; Kivic and Walne 1984; Triemer and Farmer 1991b). What is not clear is whether the vanes are elaborations of a true MTR or part of a different system altogether. Regardless, these taxa may possess important intermediate states for interpreting the evolution of a feeding apparatus of rods and vanes. It is important to note

that observations of other members in the genera, namely *C. robusta* and *P. cantuscygni*, possess a distinct MTR without evidence of vanes or rods (Triemer and Farmer 1991a, 1991b). The possibility of discovering intermediate states for the feeding apparatus and of resolving some of these inconsistent observations will hopefully inspire further ultrastructural exploration of more phagotrophic euglenids.

Dinema and the origin of the plastic pellicle

A feeding apparatus of rods and vanes first evolved in obligate bacteriotrophs with rigid pellicles composed of 10–12 longitudinally arranged strips (Fig. 14–16a). In these taxa (clade **D**) and in *Dinema*, the rods extend along the entire length of
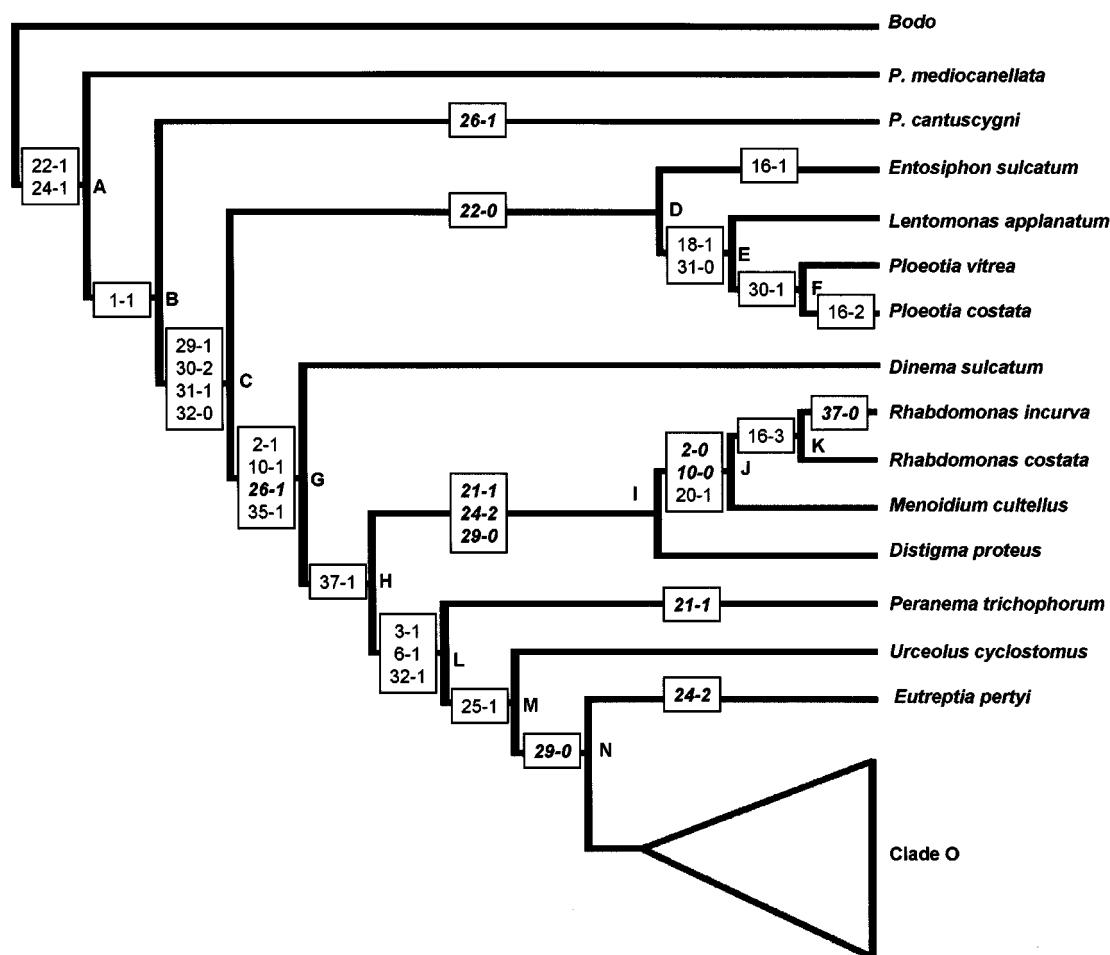

Fig. 15. Many of the morphological character states listed in Table 2 have been parsimoniously mapped onto the tree topology shown in Fig. 14. Two numbers separated by a dash represent each apomorphy; the first refers to the character and the second refers to the respective character state (Tables 2 and 3). Homoplastic character states are shown in bold and italics. Letters correspond to the clades indicated in Fig. 14.

relatively small cells (≤ 20 μm). However, a number of key innovations occurred in the most recent common ancestor of clade **G** that permitted euglenids to exploit prey much larger and nutritionally richer than bacteria (Fig. 15–16a); these evolutionary events were directly responsible for the origin of eukaryotrophy and, ultimately, phototrophy.

Among the most significant innovations was a permanent strip duplication event, where P jumped from around 10 to 20 (Fig. 16a). The strip-doubling event was likely to have been the result of a cell that duplicated its cytoskeleton without ever undergoing cytokinesis (Leander and Farmer 2001b). There were two important cytoskeletal consequences from this duplication event: (1) twice the number of articulation zones between strips was produced and (2) the strips became heli-

cally arranged. "Sliding" of adjacent strips at the articulation zones has been shown to facilitate euglenoid movement (Gallo and Schrével 1982; Suzaki and Williamson 1985, 1986). Consequently, an increase in the number of articulation zones generated a less constrained pellicle and an increased ability for changing cell shape. The length and substructural morphology of strips has been shown to remain constant during euglenoid movement (Suzaki and Williamson 1985, 1986). Therefore, in order for contractions in cell shape to occur, it is physically necessary that the strips become helically arranged. The gain of pellicle plasticity is inferred to be physically necessary for the engulfment of eukaryotic prey.

At the time eukaryotrophy became established, a beta-1, 3 glucan storage product in the form of
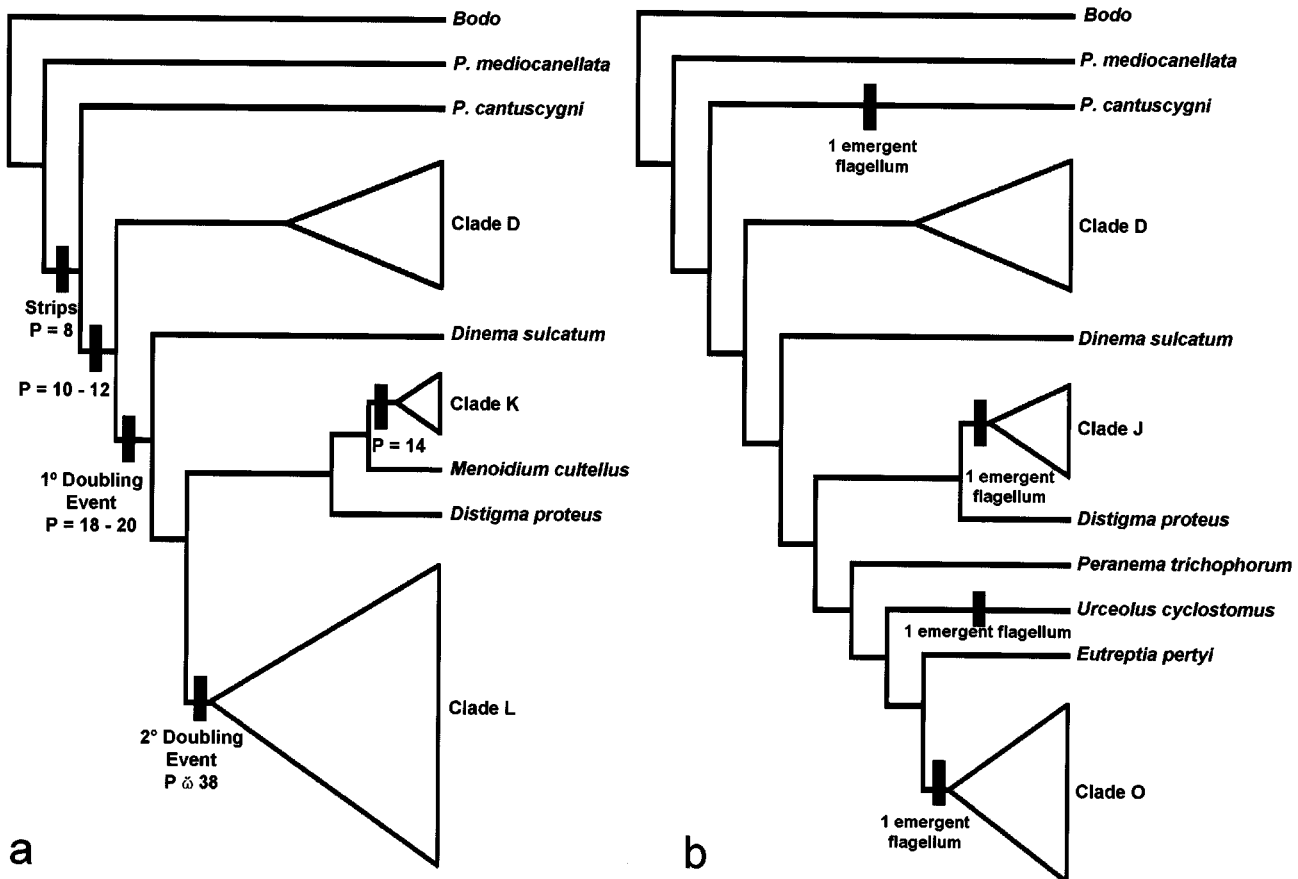

Fig. 16. Two specific examples of character evolution within the heterotrophic euglenids. **a.** Illustration showing the evolution of "P", the maximum number of strips around the cell periphery. The ancestral state for P = 8–12 and subsequently doubled to P = 18–20. A second strip-doubling event, where P jumped from 20 to about 40, occurred prior to the ancestor of clade **L**. Reduction in the number of strips evolved convergently in the ancestor of clade **K** (P = 14) and within clade **L** (Leander and Farmer 2001b). **b.** Illustration showing convergent evolution in the number of emergent flagella. All taxa possess two emergent flagella except where indicated. Letters correspond to the clades in Fig. 14.

paramylon grains emerged that has been retained in all taxa of clade **G** (Fig. 14). There are no confirmed reports of paramylon in kinetoplastids or the phagotrophic euglenids that diverge prior to clade **G**. Beta-1, 3 glucans are usually found in groups of phototrophic eukaryotes such as dinoflagellates, chrysophytes, and haptophytes (Leedale 1967; Kivic and Walne 1984; Kiss et al. 1987, 1988). Both the eukaryotrophic ancestry and exclusive presence of paramylon in clade **G** feeds speculation that the paramylon grains of euglenids were derived from non-chlorophyte phototrophic prey. If this did occur, then presumably the genetic machinery necessary for maintaining paramylon grains was transferred from the prey to the host's genome.

An apparent trend in euglenid evolution is the tendency for fairly obvious structures to become independently lost multiple times. This appears to have happened to (1) the feeding apparatus of rods

Fig. 17. Hypothetical scenario for the evolution of strips within clade **I** (Fig. 14); see text for discussion. **a.** *Dinema sulcatum;* illustration represents those strips positioned to the left of the flagellar strip as viewed from the posterior end (Fig. 6). **b.** *Distigma proteus.* **c.** *Distigma curvatum* (Angeler et al. 1999). **d.** *Distigma elegans* and *D. sennii* (Angeler et al. 1999). **e.** *Menoidium cultellus.* **f.** *Rhabdomonas costata* and *R. incurva.* Arrows indicate homologous heel-like structures marking the articulation zones between fused strips.

and vanes within clade **C**, (2) chloroplasts within clade **N**, (3) an emergent ventral flagellum within clade **B** (Fig. 16b), and (4) the flagellar strip within clade **G** (Fig. 14–15). Accordingly, it would be poor taxonomic practice to group euglenid taxa based on the absence of any of these features. We suspect that the ancestor of clade **G** possessed a distinct flagellar strip, like those found in *Dinema* and *Peranema* (Roth 1959; Hilenski and Walne 1985a) (Fig. 5–8), that was lost independently in the ancestors of clades **I** and **M**. The flagellar strips of these eukaryotrophs may be homologous to a strip on the distinct ventral surface of pellicles found in clade **E** (Fig. 14) (Triemer 1986; Farmer and Triemer 1988b, 1994) and perhaps also to the ventral grooves of some bodonids. The flagellar strip may be an important marker for following the ontogenetic and phylogenetic development of the euglenid cytoskeleton. There might be a permanent association between the ventral flagellum and the flagellar strip that can be traced in all lineages of euglenids. Identification of this specialized strip in various taxa may help delineate strips resulting from early evolutionary events (e.g., permanent strip duplication events) from those derived from relatively late events (Leander and Farmer 2001b).

Rhabdomonads and distigmids have a eukaryotrophic ancestry

Based primarily on the split-ringed elements or "scrolls" surrounding the canal and the absence of a feeding apparatus, Leedale and Hibberd (1974) proposed the "Rhabdomonadales". Subsequent investigation by Cann (1986) showed that taxa other than *Rhabdomonas* and *Menoidium* (e.g., *Gyropaine*, *Parmidium*, and *Rhabdospira*) also possess the unusual supporting elements around the canal. Rhabdomonads also possess rigid pellicles with fused strips that are often arranged longitudinally (Fig. 1d–e); this presents the possibility that the group is derived directly from rigid bacteriotrophs such as *Ploeotia* and *Entosiphon*.

However, we have demonstrated that *D. proteus* also possesses split-ringed structures around the canal (Fig. 11) despite previous ultrastructural studies that did not report the presence of these structures (Yamaguchi and Anderson 1994; Angeler et al. 1999). Moreover, our cladistic analysis of morphological characters grouped *Distigma* with the rhabdomonads (Fig. 11 and 14), which is con-
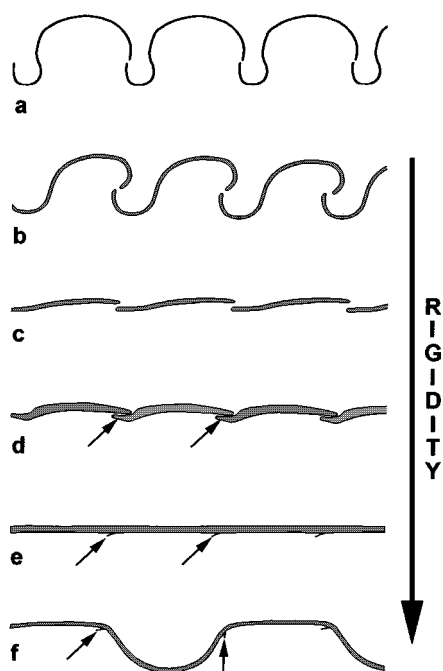
sistent with the finding of Müllner et al. (2001) and Preisfeld et al. (2001) using small subunit rDNA sequences. Clade **I** was rather surprisingly derived from within the eukaryotrophs (Fig. 14). Because distigmids are capable of intense euglenoid movement, which originated in the ancestor of clade **G** (Fig. 14–15), we can more confidently infer that rhabdomonads were derived from plastic, eukaryotrophic ancestors. We did not find any additional evidence, however, suggesting that the scrolls around the canal were derived from a feeding apparatus of rods and vanes (Leedale and Hibberd 1974; Leedale 1978).

In the context of this new insight, we can amend the scenario for the evolution of strip substructure presented by Leander and Farmer (2001a) (Fig. 17). We infer that sigmoidal strips like those found in *D. proteus* became increasingly flattened so that the arch and heel became more or less indistinguishable as in *D. curvatum* (Angeler et al. 1999) (Fig. 17). The frames began to thicken leaving a thin hook-like region that extended prearticularly beneath the overhang of an adjacent strip as in *D. elegans* and *D. sennii* (Angeler et al. 1999). We suspect that these hook-like regions are homologous to the delicate structures marking the fused articulation zones (syn. discontinuities, Leedale and Hibberd 1974) of *Menoidium* and *Rhabdomonas* (Leander and Farmer 2001a) (Fig. 17). The peculiar strip doublets of *Rhabdomonas* and the completely fused strips of *Parmidium* and *Gyropaine* (Cann 1986) are inferred to have evolved independently from strips similar to *Menoidium*.

The number of emergent flagella changed from two to one during the evolution of rhabdomonads from the ancestor of clade **H** (Fig. 16b). *Distigma proteus* possesses two emergent flagella, but the rather short ventral flagellum may indicate an intermediate step toward a single emergent flagellum. Moreover, there are several taxa currently classified as *Astasia* that are most likely distigmids with a single emergent flagellum (e.g., *A. edax* and *A. concinna*, Christen 1959; *A. tortuosa*, Angeler 1998; *A. comma* and *A. curvata*, Müllner et al. 2001). If these taxa are truly misplaced as small subunit rDNA sequences suggest (Müllner et al. 2001), then they provide additional intermediate states between the biflagellated distigmids and the uniflagellated rhabdomonads. Detailed analyses of the pellicle of these "*Astasia*" taxa, particularly the value of P (the number of strip around the cell periphery) and W_P (the number of whorls of strip re-

duction), will more confidently demonstrate their relationship to distigmids (Angeler 1998; Angeler et al. 1999; Leander and Farmer 2000a); distigmids possess P = 18—20 and W_P = 0, whereas *Astasia* (e.g., *A. longa*) possesses P = 40 and W_P = 3 (Leander and Farmer 2000a).

The idea that *Distigma*, *Eutreptia*, and *Eutreptiella* should be classified together within an order "Eutreptiales" is inconsistent with both molecular and morphological data (Müllner et al. 2001; Preisfeld et al. 2001) (Fig. 14). A new taxon name should probably be erected that encompasses true distigmids with the rhabdomonads. This group may be diagnosed as possessing split-ringed elements around the canal (scrolls), P = 18, W_P = 0, osmotrophy, and a dorsal flagellum with a highly mobile beat pattern. It is currently unclear whether the supporting elements around the canal of *P. trichophorum* (Fig. 9) are homologous with the scrolls of distigmids and rhabdomonads; we have mapped them as being analogs (Fig. 15), but the origin of scrolls in the ancestor of clade **H** and their subsequent loss in clade **M** is an equally parsimonious scenario.

Urceolus cyclostomus and the origin of phototrophs

A number of key evolutionary innovations occurred in the ancestor of clade **L** that set the stage for the endosymbiotic origin of phototrophy in euglenids (Fig. 14–15). The most significant of which was a second permanent strip duplication event that increased the number of strips around the cell periphery from 20 to about 40 (Fig. 16a). Associated with this event were increases in the cell size (≥ 20 µm) and the number of articulation zones. The rods of the feeding apparatus became localized to the anterior portion of the cell, where the length of the rods was less than 1/2 the length of the cell. The phagotrophic taxa within clade **L** became voracious predators of diverse eukaryotic prey, including phototrophic euglenids (Triemer 1997).

Another important correlation to the second strip duplication event was the reinforcement of the canal by strips. Canals lined by strips have only been found in taxa within clade **L** (Fig. 14–15). In most phototrophs, the number of strips surrounding the canal lumen, C, is half the number around the cell periphery, P (Leander and Farmer 2000a, 2000b, 2001b). In *P. trichophorum* and *U. cyclosto-*

mus, P was equivalent to C (Fig. 12). At the posterior end of phototrophs, terminating strips are organized as distinct whorls (Leander and Farmer 2000a, 2000b, 2001b). Patterns of strip reduction are also present at the posterior end of *P. trichophorum* (Leander and Farmer 2001b) and *U. cyclostomus*, however the current absence of SEM data makes the exact organization of terminating strips on *U. cyclostomus* indeterminable. Nonetheless, the presence of strips around the canal, the presence of P ≥ 38, and the presence of posterior strip reduction are important cytoskeletal evidence linking the phototrophs to eukaryotrophic ancestors.

Because phototrophs are the only euglenids known to possess a photoreception apparatus, perhaps the most significant discovery linking the phototrophs to eukaryotrophs is the presence of a presumptive paraflagellar swelling and stigma in *U. cyclostomus* (Fig. 13). Presumably, the stigma and paraflagellar swelling of *U. cyclostomus* function together in photoreception in much the same way these structures function in phototrophs (Robenek and Melkonian 1983; Kuźnicki et al. 1990). It has been generally argued that a photoreception apparatus should logically develop after the acquisition of plastids because the necessity for the new photosynthetic host to find optimal light would become a strong selective pressure (Kivic and Walne 1983). However, the possession of both a feeding apparatus of rods and vanes and a photoreception apparatus within the same euglenid cell may be explained by one of three alternative scenarios: (1) the apparatus in *U. cyclostomus* is not homologous to the photoreception apparatus in phototrophs, (2) the photoreception apparatus evolved after or at the same time as plastids, but plastids have subsequently been lost in *U. cyclostomus*, or (3) the photoreception apparatus evolved before the origin of plastids and is homologous in all euglenids that possess one.

Our cladistic analysis and the identical structure and cellular position of the apparatus (e.g., not associated with the plastid as in most other groups of algae) in both *U. cyclostomus* and phototrophs rules out the first scenario. The fact that *U. cyclostomus* lacks any evidence of a plastid, possesses a feeding apparatus of rods and vanes and actively engages in eukaryotrophy seems to rule out scenario two. The third scenario implies that a eukaryotroph benefited from the capability of photoreception. The most obvious advantage is that eukaryotrophs capable of photoreception (e.g.,

phototaxis) would have an increased ability to position themselves in an environment with abundant phototrophic prey. It makes perfect sense, then, that the first eukaryotrophic euglenid to consume and subsequently foster an endosymbiotic relationship with green algal plastids already possessed the photoreception apparatus. The presence of the apparatus in the host may have actually increased the likelihood that the endosymbiosis would ultimately succeed. Nonetheless, it is still unclear whether the stigma of *U. cyclostomus* originated autogenously or was derived from an independent endosymbiotic event that occurred before plastid acquisition.

Menoidium and *Peranema* have "layered material" near the base of the dorsal flagellum that coincides with the position of the paraflagellar swelling in other taxa (Leedale and Hibberd 1974; Hilenski and Walne 1985b). Leedale and Hibberd (1974) suggested that this material might be evidence of a vestigial photosensory swelling indicating that rhabdomonads were derived from phototrophs. The layered material is positioned on the same side of the flagellum as the paraxial rod, and the distinction between the two structures is extremely subtle. We infer that the layered material of *Menoidium* and *Peranema* is not homologous to the paraflagellar swelling of *U. cyclostomus* because the latter is significantly more electron dense (like those in phototrophs) and positioned on the opposite side of the paraxial rod. However, if the layered material is homologous to paraflagellar swellings, than we would argue based on our cladistic analysis that the material represents an intermediate state in the evolution of photosensory swellings and does not represent a vestige of phototrophic ancestry.

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