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Reconstruction of the feeding apparatus in *Postgaardi mariagerensis* provides evidence for character evolution within the Symbiontida (Euglenozoa)

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Abstract

Microbial eukaryotes living in low oxygen environments often have novel physiological and morphological features that facilitate symbiotic relationships with bacteria and other means for acquiring nutrients. Comparative studies of these features provide evidence for phylogenetic relationships and evolutionary history. *Postgaardi mariagerensis*, for instance, is a euglenozoan that lives in low oxygen environments and is enveloped by episymbiotic bacteria. The general ultrastructure of *P. mariagerensis* was described more than a decade ago and no further studies have been carried out since, mainly because these cells are difficult to obtain. *Postgaardi* lacks the diagnostic features found in other major euglenozoan lineages (e.g., pellicle strips and kinetoplast-like mitochondrial inclusions) and no molecular data are available, so the phylogenetic position of this genus within the Euglenozoa remains unclear. We re-examined and reconstructed the ultrastructural organization of the feeding apparatus in *Postgaardi* by serial sectioning an existing block of resin-embedded cells. *Postgaardi* possesses distinctive finger-like projections within the feeding apparatus; this system has only been found in one other highly distinctive flagellate, namely the symbiontid *Calkinsia*. Detailed comparisons of the cytoskeleton in *Postgaardi* and in two symbiontids, *Calkinsia* and *Bihospites*, provided new evidence for phylogenetic relationships and character evolution in all three genera.

Keywords: Anaerobe; Euglenida; Euglenozoa; Symbiontida; Symbiosis; Uncultured protist

Introduction

Low-oxygen environments contain a substantial number of poorly studied taxa of microbial eukaryotes; many of these have been detected only through environmental DNA surveys of microbial diversity (Alexander et al. 2009; Dawson and Pace 2002; Stoeck and Epstein 2003; Stoeck et al. 2003; Takishita et al. 2010, 2005, 2007a,b; Wylezich and Jurgens 2011). By contrast, some microbial eukaryotes

Abbreviations: AL, anterior lip; FdP, feeding pocket; FP, flagellar pocket; G, gutter; M, mound in gutter; MTR, reinforced band of microtubules; R, reinforced ridge.

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living in low oxygen environments have been observed directly under light microscopy, but described without DNA sequence information and only with drawing or light micrographs (Bernard et al. 2000; Fenchel and Finlay 1995). The inherent challenges associated with anaerobic cultivation make data on these microbial eukaryotes difficult to obtain, especially ultrastructural data using transmission electron microscopy (TEM). The limited information for many microbial eukaryotes living in low oxygen environments combined with the uncoupling of microscopy data from DNA sequence information hinders inferences about their biology and phylogenetic positions within the overall tree of eukaryotes.

Postgaardi mariagerensis Fenchel, Bernard, Esteban, Finlay, Hansen & Iversen, 1995 is a flagellate with episymbiotic bacteria on the surface of the cell and is reported from two low-oxygen water columns: a Danish fjord and Burton Lake, Antarctica (Fenchel et al. 1995; Simpson et al. 1996/1997). Data from light and electron microscopy studies of these field samples have demonstrated that this organism possesses all of the diagnostic traits of the Euglenozoa, a major group of microbial eukaryotes whose members have diverse modes of nutrition, including phagotrophy, mixotrophy, phototrophy and parasitism (Leander 2004; Simpson 1997; Yamaguchi et al. 2012). *Postgaardi*, however, lacks the traits that diagnose the major euglenozoan subgroups (euglenids, diplonemids, and kinetoplastids) and has been treated as incertae sedis within the Euglenozoa (Simpson et al. 1996/1997).

The Symbiontida is a recently described subgroup of the Euglenozoa that contains *Calkinsia* and *Bihospites*, both of which live in low oxygen environments and have episymbionts that are very similar to those on *Postgaardi* (Breglia et al. 2010; Yubuki et al. 2009). The possibility of a close relationship between *Postgaardi* and members of the Symbiontida has been discussed in previous studies, but compelling evidence for this affinity is currently unavailable (Breglia et al. 2010; Yubuki et al. 2009). Despite the absence of any DNA sequences generated directly from isolated cells of *Postgaardi*, there are several environmental DNA sequences in GenBank that cluster within the Symbiontida, and it has been speculated that these might represent *Postgaardi* (Breglia et al. 2010; Edgcomb et al. 2011; Yubuki et al. 2009).

Cells of *Postgaardi* are very difficult to obtain and living cultures do not exist. In order to more rigorously infer the phylogenetic position of *Postgaardi*, we reinvestigated resin-embedded cells collected from Burton Lake, Antarctica (originally reported in Simpson et al. 1996/1997). Using serial sectioning, we comprehensively reconstructed the ultrastructural details associated with the feeding apparatus in *Postgaardi*, previously described as a "complex depression" (Simpson et al. 1996/1997). Comparisons of the feeding apparatus in *Postgaardi* and *Calkinsia* uncovered putative synapomorphies that support the phylogenetic position of *Postgaardi* within the Symbiontida.

Material and Methods

Transmission electron microscopy

The resin block examined in this study was prepared from a sample of *Postgaardi* cells collected from Burton Lake, Antarctica, as reported by Simpson et al. (1996/1997). Serial ultrathin sections were cut on a Leica EM UC6 ultramicrotome and double stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963). The serial sections were observed using a Hitachi H7600 electron microscope.

Results and Discussion

The general ultrastructural features of *Postgaardi mariagerensis* observed here were consistent with the previous report in Simpson et al. (1996/1997), and we adopted the same structural terminology and abbreviations. One important exception is that we distinguished the more specific term "feeding pocket" (FdP) from the broader term "feeding apparatus" (FA) as used in Simpson et al. (1996/1997). Nonetheless, we focused our study on details of the entire feeding apparatus, which was positioned on the right posterior side of the flagellar pocket. The feeding apparatus consisted of four main elements: (1) an elongated oval-shaped gutter (G) surrounding an elongated mound; (2) a reinforced ridge (R) that extended over the left side of the gutter; (3) an anterior lip (AL) that extended over the right side of the gutter and overlapped the distal edge of the reinforced ridge; and (4) a feeding pocket (FdP) located in the left portion of the gutter (Figs 1, 2). Much of the gutter is supported by a band of five separated microtubules that represents a portion of the ventral microtubular root, and that supports other elements of the feeding apparatus (see below).

Transverse sections through the middle part of the feeding apparatus demonstrated a W-shaped depression consisting of the central mound between the right and left grooves of the elongated oval-shaped gutter (Figs 2–7). The feeding pocket extended deeper into the cell, and is supported by the distal portions of the band of five microtubules (Figs 6–9). The anterior lip (AL) on the right side of the gutter overlapped the reinforced ridge (R) on the left side, which together formed a canopy over the middle region of the gutter (Figs 2, 5–8). The anterior and posterior regions of the gutter were not covered by the anterior lip and reinforced ridge and were therefore exposed to the outside (Figs 1–4, 9, 10). Transverse sections through the anterior region of the gutter showed a relatively shallow central mound (Fig. 3). In more posterior regions of the feeding apparatus, the left and right grooves of the gutter became deeper and the mound became more pronounced (W-shaped, Fig. 6). The mound became wider and more flattened at the posterior-most region of the gutter (Figs 7–9). Six finger-like projections were positioned in the right wall of the anterior-most region of the gutter

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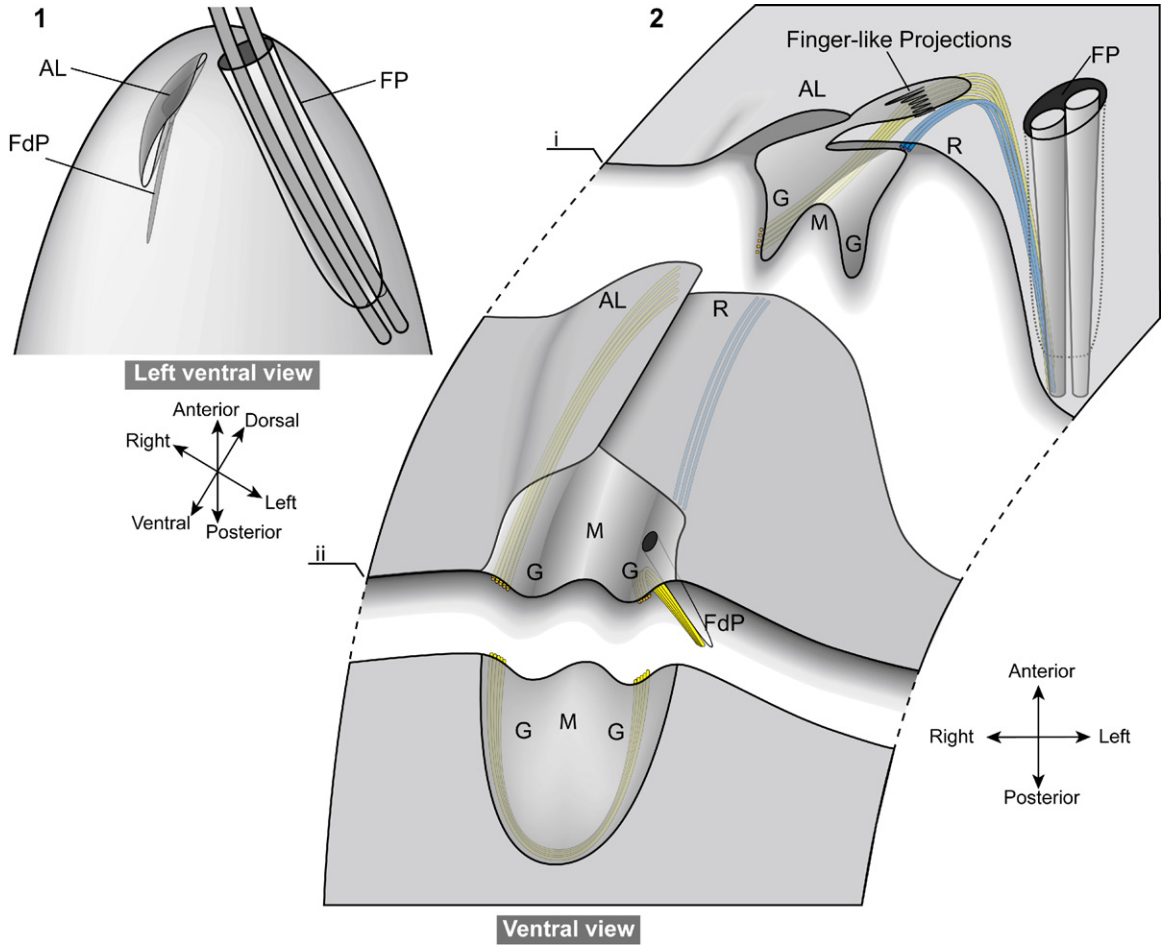

Figs 1, 2. Reconstruction of the feeding apparatus in *Postgaardi mariagerensis* from ultra-thin serial TEM sections. Episymbiotic bacteria on the cell surface are not shown for the sake of increased clarity. **(1)** Illustration showing the relative locations of the flagellar pocket (FP) and the feeding apparatus consisting of an anterior lip (AL) and a feeding pocket (FdP). **(2)** Illustration showing two transverse sections (i and ii) through the main elements of the feeding apparatus. The anterior lip (AL) covers the right side of an elongated oval-shaped gutter (G) with a central mound (M); the anterior lip also covers the distal edge of a reinforced ridge (R) that covers the left side of the gutter. The feeding pocket (FdP) is positioned within the left groove of the gutter. Two bands of microtubules originate from the ventral root of the flagellar apparatus. A band of three microtubules (blue) supports the reinforced ridge; a band of five microtubules (yellow) supports finger-like projections in the anterior region of the gutter and passes along the right wall and the poster region of the gutter before turning inward to reinforce the feeding pocket (FdP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

(Figs 2–5, 11–16). The finger-like projections were supported at their bases by the band of five microtubules and densely stained fibrous material. The projections themselves were supported by extensions of this fibrous material.

In addition to the band of five microtubules associated with the right wall of the gutter, a band of three microtubules was associated with fibrous material in the reinforced ridge; both bands were linked to the ventral root of the flagellar apparatus (Figs 2–7, 13–16). The presence of eight microtubules in total was the same number of microtubules in the proximal portion of the ventral root reported by Simpson et al. (1996/1997). The microtubules from the ventral root extended anteriorly from alongside the long basal bodies and towards the surface of the cell before separating into the distinct five-microtubule and three-microtubule bands (Figs 2, 15–17). The band of five microtubules reinforced the six finger-like projections, extended down the right side of the gutter, and encircled the posterior region of the gutter before moving inward with the feeding pocket in the left groove of the gutter (Figs 2–10; see also Figs 20, 22 of Simpson et al. 1996/1997). The band of three microtubules was located within the base of the reinforced ridge and supported the upper-left wall of the gutter (Figs 2–7, 16).

Phylogenetic position of *Postgaardi*: character evolution within the Symbiontida

The ultrastructural reconstruction of the feeding apparatus in *Postgaardi* supports the hypothesis that *P. mariagerensis*, *Calkinsia aureus* and *Bihospites bacati* are all closely related to one another (Breglia et al. 2010; Yubuki et al.

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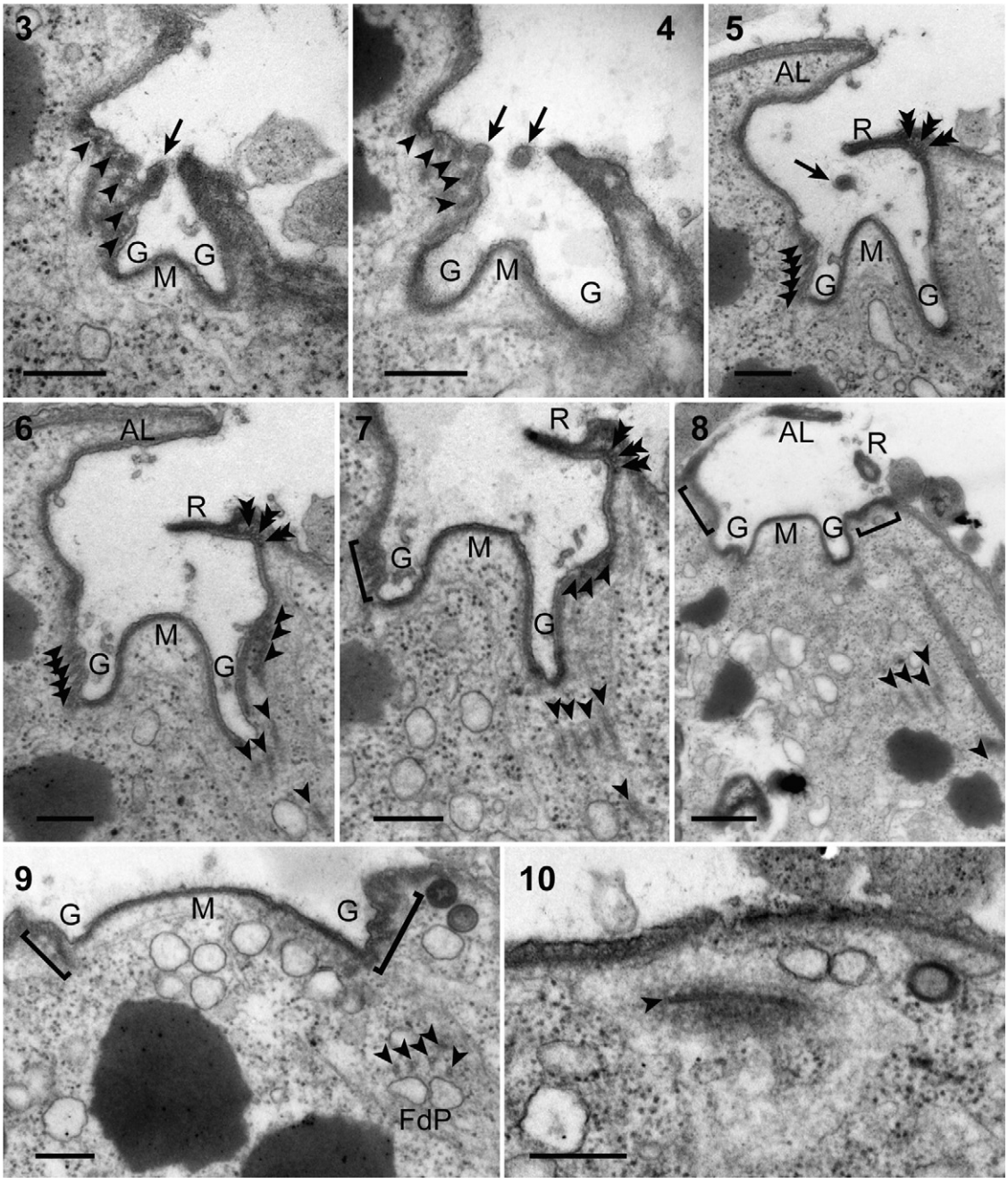

Figs 3–10. Transverse TEM sections through feeding apparatus of *Postgaardi mariagerensis*. Scale bars = 500 nm. **(3–8)** Non-consecutive serial sections (anterior to posterior) through the feeding apparatus showing fibrous finger-like projections (arrows) and microtubules embedded in fibrous material (arrowheads/square brackets). An anterior lip (AL) from the right side of the gutter (G) covers the reinforced ridge (R) from the left side of the gutter (G). A band of three microtubules (double arrowheads) reinforces the ridge (R). Note that only three microtubules (arrowheads) are shown on the left side of the gutter on **6** and **7** because the other two microtubules curve later than these three along the feeding pocket. The microtubules on the left side of the gutter on **6** and **7** are cut through twice. Accordingly, the total number of arrows is seven or eight, but not ten. The square bracket in **7** and **8** marks the band of five microtubules. **(9)** TEM section through the posterior regions of the feeding apparatus showing the absence of the anterior lip and the reinforced ridge. The feeding pocket (FdP) and associated microtubules (arrowheads) were visible in cross section. The square brackets mark the band of five curved microtubules positioned on the both sides of the gutter. **(10)** Tangential TEM section through the posterior edge of the gutter showing the curved microtubule (arrowhead).

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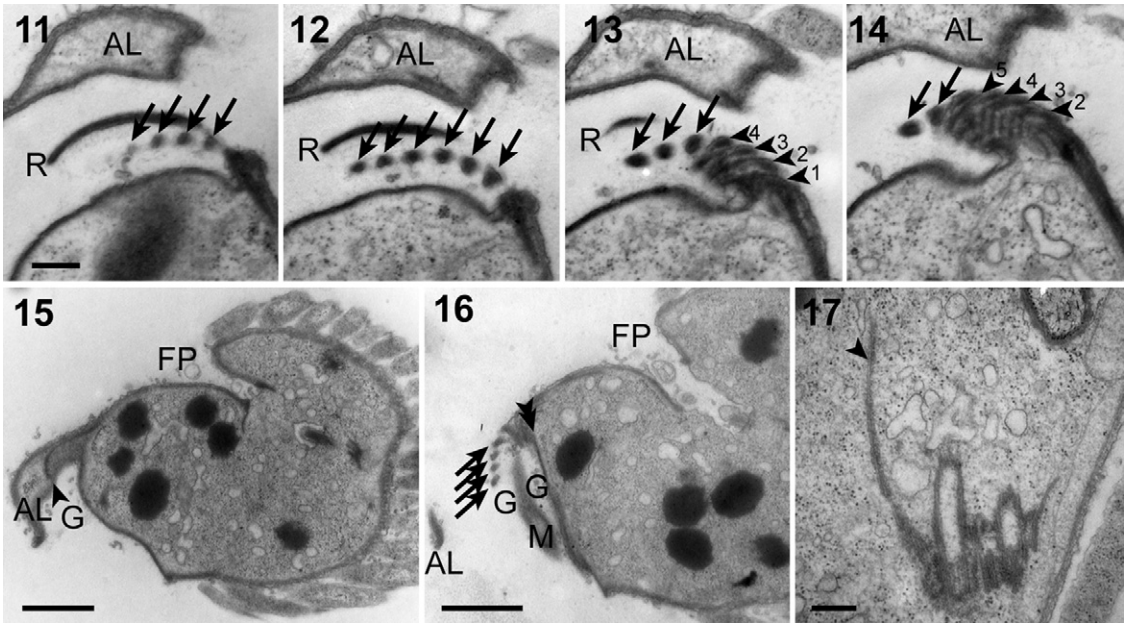
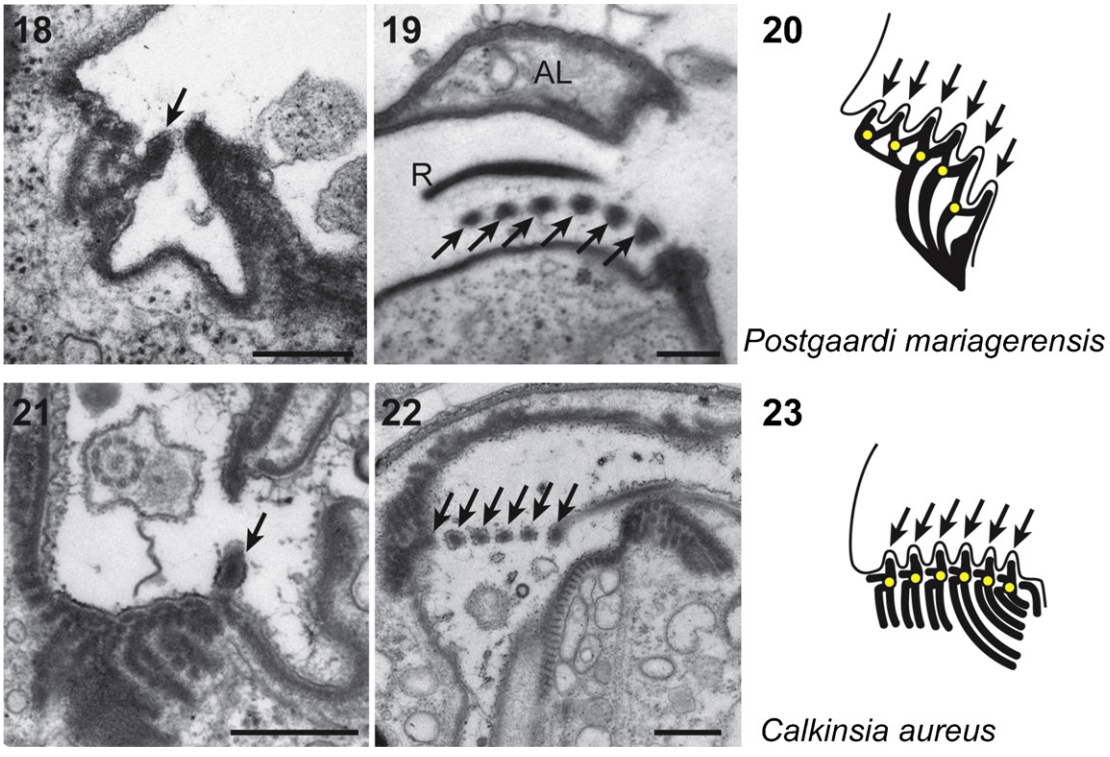


Figs 11–17. TEM images of *Postgaardi mariagerensis*. **(11–14)** Non-consecutive serial sections through the finger-like projections (arrows) in the anterior region of the feeding apparatus in *Postgaardi mariagerensis*. Five microtubules (arrowheads) are associated with the finger-like projections (arrow). Scale bars = 500 nm. **(15, 16)** TEM sections through the flagellar pocket (FP) and the anterior region of the feeding apparatus showing a band of five microtubules (arrowheads) and a band of three microtubules (double arrowheads). **(17)** Longitudinal section showing two long basal bodies and the ventral root (arrowhead). Scale bars = 2 μm. AL, anterior lip; FP, flagellar pocket; G, gutter; M, mound; R, reinforced ridge.



Figs 18–23. Comparison of the near identical configuration of finger-like projections found in *Postgaardi mariagerensis* **(18–20)** and *Calkinsia aureus* **(21–23)**. Microtubules are shown in yellow **(20, 23)**. AL, anterior lip; arrows, finger-like projections; R, reinforced ridge. Scale bars = 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

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2009). All three genera have episymbionts on the cell surface and mitochondrion-derived organelles under the cell membrane (Breglia et al. 2010; Edgcomb et al. 2011; Simpson et al. 1996/1997; Yubuki et al. 2009). *Bihospites* has S-shaped (pellicle) surface folds and a robust feeding rod, which are similar to the traits found in phagotrophic euglenids (Breglia et al. 2010). Moreover, *Bihospites*, *Postgaardi*, *Calkinsia* and many phagotrophic euglenids have relatively long basal bodies, a trait that is not present in most kinetoplastids and diplonemids. Although the presence of S-shaped folds and a robust feeding rod in *Bihospites* suggests a close affiliation with the Euglenida, these traits are not present in either *Calkinsia* or *Postgaardi*. Instead, *Calkinsia* forms an elaborate (orange) extracellular matrix and has a highly reduced feeding rod; *Postgaardi* lacks an elaborate extracellular matrix and has a novel oval-shaped gutter covered by an anterior lip that overlaps a reinforced ridge. Nonetheless, the distinctive array of six finger-like projections observed here in the feeding apparatus of *Postgaardi* has only been observed in one other eukaryote so far, namely *Calkinsia* (Figs 18–23; Yubuki et al. 2009). Therefore, we interpret the distinctive array of six finger-like projections as a putative synapomorphy that evolved a common ancestor of *Postgaardi* and *Calkinsia*. *Postgaardi* and *Calkinsia* also both have a dense central element in the flagellar transitional zone that does not extend into the basal body, a configuration that is unlike the transitional zone of *Bihospites*, where there is a much thicker core element restricted to the basal bodies (Fig. 17; Fig. 11B of Breglia et al. 2010; Fig. 17 of Simpson et al. 1996/1997; Fig. 6 of Yubuki et al. 2009). Some phagotrophic euglenids, such as *Notosolenus* (*Petalomonas*) *mediocanellata* and *Lentomonas* (*Entosiphon*) *applanatum*, have a dense element in the flagellar transition zone as well (Farmer and Triemer 1988, 1994; Triemer and Farmer 1991); this feature provides additional support for a close relationship between euglenids and symbiontids.

Molecular phylogenetic analyses of small subunit (SSU) rRNA gene sequences demonstrate that *Bihospites* and *Calkinsia* group together with robust statistical support as a well-supported subclade within the Euglenozoa, namely the Symbiontida (Breglia et al. 2010; Yubuki et al. 2009). These analyses also demonstrate that *Calkinsia* forms the nearest sister lineage to a clade of environmental SSU rDNA sequences isolated from low oxygen environments from all over the world; *Bihospites* forms the nearest sister lineage to the clade consisting of *Calkinsia* and these environmental sequences (Breglia et al. 2010; Edgcomb et al. 2011; Yubuki et al. 2009). By coupling this molecular phylogenetic context with the possible synapomorphies between *Calkinsia* and *Postgaardi* discussed above, we can predict with more confidence that one or more of the environmental sequences within the sister clade to *Calkinsia* might represent *Postgaardi* (Fig. 24). Obviously, sequence data from *Postgaardi* would be required to test this hypothesis.

The proposed close phylogenetic relationship of *Postgaardi* and *Calkinsia* is consistent with a broader phylogenetic pattern across eukaryotes whereby the anaerobic members of major taxa tend to form into one or a few distinct clades, rather than representing many distinct lineages (e.g., archamoebae, metamonads, anaerobic heteroloboseans) (Barberá et al. 2007; Hampl et al. 2005, 2009; Pánek et al. in press). It also suggests straightforward evolutionary scenarios for morphological evolution within the Symbiontida as a whole. For instance, pellicle strips, feeding rods and long basal bodies could be inferred to have been present in the most recent euglenid ancestor of symbiontids before the incremental reduction (and loss) of feeding rods and pellicle folds in common ancestors of *Calkinsia* and *Postgaardi* (Fig. 24). The reduced feeding rod similar to that seen in *Calkinsia* would then have been completely lost in *Postgaardi*, in association with the evolution of the novel feeding apparatus we describe here (Figs 1–2, 24).

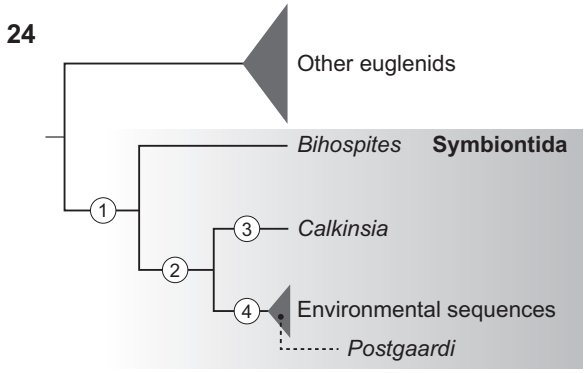

Fig. 24. A possible history of character evolution within the Symbiontida as inferred from available ultrastructural data and molecular phylogenetic analyses of small subunit rRNA gene sequences (the equation of *Postgaardi* and the 'environmental sequences' clade is conjectural). Position 1: Low oxygen habitat, episymbiotic bacteria, mitochondrion-derived organelles immediately beneath the cell surface. Position 2: An array of six finger-like projections in the anterior region of the feeding apparatus, dense element in flagellar transitional zone, reduced feeding rods, and the loss of pellicle strips. Position 3: An elaborate (orange) extracellular matrix with conduits beneath the episymbiotic bacteria, an extrusomal pocket. Position 4: A feeding apparatus consisting of an elongated oval shaped gutter covered by an anterior lip overlapping a reinforced ridge, the loss of feeding rods.

Remarks on the systematics of *Postgaardi mariagerensis*

Simpson et al. (1996/1997) classified *Postgaardi mariagerensis* as incertae sedis within the Euglenozoa. Cavalier-Smith (1998) subsequently proposed a taxonomic scheme for the Euglenozoa that placed *Postgaardi mariagerensis* within the monospecific class Postgaardea. In this scheme, the Euglenozoa was split into two major subgroups distinguished primarily by the type of feeding apparatus present: Plicostoma (including Diplonemia and Euglenoida) and Saccostoma (including Kinetoplastea and

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Postgaardea) (Cavalier-Smith 1998, 1999, 2002, 2003). Detailed analyses of the feeding apparatus of diplonemids showed that its plicate nature was not similar to that of certain euglenids (Montegut-Felkner and Triemer 1996). Furthermore, the sister relationship between the Kinetoplastida and the Diplonemida is supported by comparative morphological and molecular phylogenetic data as well as the shared possession of U-insertion RNA editing in mitochondria (Flegontov et al. 2011; Kiethega et al. 2011; Marande and Burger 2007; Maslov et al. 1999; Simpson and Roger 2004; Simpson et al. 2004). Conversely, our data provide strong support for the hypothesis that *Postgaardi* is a member of the Symbiontida, which is a major clade nested within the Euglenida. This hypothesis is supported by comparative ultrastructural data and is consistent with current molecular phylogenetic data (Breglia et al. 2010). The previously proposed Plicostoma/Saccostoma classification scheme (Cavalier-Smith 1998, 1999, 2002, 2003) is therefore not supported by current evidence.

Acknowledgements

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