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Phylogenetic Placement and Morphological Description of Three Species of Heterotrophic Euglenids (Euglenozoa) From Arizona: *Scytomonas flumensis* n. sp., *Ploeotia protuberantia* n. sp., and *Hokulea pusilla* comb. nov

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ABSTRACT

Euglenids are microbial flagellates best known for their highly flexible photosynthetic species (e.g., *Euglena gracilis*), though many heterotrophic groups remain poorly characterized at both molecular and ultrastructural levels. In particular, rigid heterotrophs such as ploeotids and petalomonalads are highly diverse, yet most described species were documented only with light microscopy, largely because they are difficult to cultivate. Here, we establish cultures for three new isolates of ploeotids and petalomonalads (*Hokulea pusilla* comb. nov., *Ploeotia protuberantia* sp. nov., *Scytomonas flumensis* sp. nov.) and examine them using light microscopy, scanning electron microscopy, and 18S rDNA phylogenetic analyses. These data support the recognition of two new species, *Ploeotia protuberantia* sp. nov. and *Scytomonas flumensis* sp. nov., and justify the transfer of *Ploeotia pusilla* to *Hokulea pusilla* comb. nov. based on its morphology and its affiliation with *Hokulea waialensis* in 18S rDNA phylogenies.

1 | Introduction

Euglenids (Euglenozoa) are a group of eukaryotic flagellates that occupy a wide range of environments and employ diverse nutritional modes, including phagotrophy, osmotrophy and phototrophy (Leander et al. 2017). A major synapomorphy of euglenids is the pellicle, which consists of proteinaceous strips that run beneath the plasma membrane and are subtended by microtubules (Lax et al. 2019, 2021, 2023; Lax and Simpson 2020; Leander and Farmer 2000, 2001; Leander, Triemer, and Farmer 2001; Leander, Witek, and Farmer 2001; Leander et al. 2017; Sommer and Blum 1965). The number of pellicle strips varies drastically

among euglenid groups, ranging from as few as 4 strips to more than 120 strips per cell (Esson and Leander 2008; Leander 2004; Leander et al. 2007, 2017). The pellicle determines how rigid or flexible a euglenid cell is, with cell rigidity broadly correlated to the number and arrangement of strips (Leander et al. 2017). In taxa with fewer than 20 strips (petalomonalads and ploeotids), which are often—though not always—longitudinally arranged, cells are rigid (Lax et al. 2019, 2023; Leander, Triemer, and Farmer 2001; Leander, Witek, and Farmer 2001; Leander et al. 2017). Comparatively, in taxa with more than 20 strips (i.e., the spirocutes), the pellicle strips slide past one another, allowing the cells to contract in a characteristic form of motility called

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metaboly (Leander, Triemer, and Farmer 2001; Leander, Witek, and Farmer 2001; Leander et al. 2017). Euglenids also possess a feeding apparatus that enables phagocytosis of both bacterial and eukaryotic prey, consisting of microtubular arrays associated with the vestibulum and opening into the flagellar pocket (Leander et al. 2017). The structure and organization of this apparatus differs markedly among euglenid groups. It is not a perfect correlation, but broadly speaking, euglenids with more pellicle strips (particularly those capable of metaboly) and a robust feeding apparatus are generally able to consume larger prey, particularly eukaryotes (Leander et al. 2017; Palka et al. 2025).

Euglenids comprise three major groups: the two well-resolved clades Spirocuta and Petalomonadida, and the paraphyletic 'ploetids' (Lax et al. 2023). The Spirocuta includes the phototrophic euglenids (Euglenophytes), the osmotrophic euglenids (Aphagea), and a diverse assemblage of phagotrophic euglenids (Lax et al. 2019, 2021, 2023; Lax and Simpson 2020; Lee and Simpson 2014a). The spirocutes are defined by an ancestral pellicle-strip duplication event that produced a helical, highly flexible pellicle with more than 20 strips that glide alongside one another to facilitate metaboly (Cavalier-Smith 2016; Lax et al. 2019; Leander 2004; Leander, Witek, and Farmer 2001; Leander et al. 2007; Lee and Simpson 2014a, 2014b; Paerschke et al. 2017). Petalomonads and ploetids, on the other hand, are strictly rigid and have 12 or fewer pellicle strips.

The robustly supported Petalomonadida, the sister branch to all other euglenids in multigene phylogenies, comprises phagotrophic flagellates that frequently prey on smaller organisms and glide using an anterior flagellum (Leander et al. 2017; Lee and Simpson 2014b; Lee et al. 2026). Petalomonads have a relatively simple cell morphology consisting of a rigid pellicle with 4–10 strips, one or two emergent flagella, and a simple feeding apparatus supported by a microtubule-reinforced pocket that lacks feeding rods. Almost all petalomonads are motile, with the exception of *Dolium sedentarium*, a sessile petalomonad that lacks a flagellum for at least part of its life cycle (Larsen 1990; Lax and Keeling 2023), and *Scytomonas saepesedens*, which has an attached sedentary stage for feeding (Cavalier-Smith et al. 2016).

Petalomonads are arguably the least-studied group of euglenids in terms of molecular data despite inhabiting both freshwater and marine environments, and historically, they were distinguished primarily by morphological characteristics (Lee and Simpson 2014b; Lee et al. 2026). Despite their key phylogenetic position as the deepest branch of euglenids (Lax and Keeling 2023; Lax and Simpson 2020; Lax et al. 2021; Leander 2004; Leander et al. 2007), they have remained relatively poorly characterized at both molecular and microscopic levels until recently, and multigene phylogenetic data are still available for only four genera (Lax and Keeling 2023). Consequently, early phylogenies inferred from 18S rDNA sequences are notoriously plagued by paraphyletic genera, which have only recently begun to be disentangled through 18S rDNA sequencing and ultrastructural characterization of 13 newly isolated freshwater and marine strains thereby resolving petalomonads into six distinct clades and several new genera (Lee et al. 2026). Despite this substantial advancement in our understanding of petalomonad diversity, the group remains under-characterized relative to other euglenid lineages, with

environmental sequencing surveys demonstrating profound hidden diversity within this group (Lee et al. 2026).

Lastly, the 'ploetids' are a paraphyletic assemblage of rigid heterotrophic euglenids that share several morphological traits: a conspicuous feeding apparatus with robust rods and vanes, 10–12 pellicle strips, and two flagella where one anterior flagellum consistently sweeps in front of the cell while the other (often longer and thicker) posterior flagellum facilitates gliding motility (Lax and Simpson 2013; Lax et al. 2019, 2023; Leander et al. 2007, 2017). The pellicle of these taxa contains considerable diversity in the arrangement, width and length of pellicle strips. For example, in some ploetids, these strips can be heteromorphic (e.g., narrow, recessed strips alternating with wider strips in *Serpenomonas costata*; Lax et al. 2019; Triemer and Fritz 1986) or projecting outwards like 'keels' (e.g., *Ploeotia vitrea*, *P. oblonga*, *Olkasia polycarbonata*; Palka et al. 2025). More recently, the rigid ploetid *Hokulea waialensis* has been shown to possess pellicle strips that appear helically arranged (Ruiz et al. 2026), a trait often associated with spirocutes (Leander, Triemer, and Farmer 2001; Leander, Witek, and Farmer 2001; Leander et al. 2017). The distinctive feeding apparatus of ploetids consists of two rods and four vanes that together can appear either hook-shaped, as observed mostly in alistosan ploetids, or chisel-shaped, like in *Olkasia polycarbonata* and *Ploeotia plumosa* (Lax et al. 2019, 2023; Lax and Simpson 2020; Lee and Patterson 2000; Palka et al. 2025). Ploetids represent a significant portion of euglenid diversity, including several distinct clades (e.g., Alistosa and Karavia) as well as several taxa whose placement among euglenids remains uncertain with the limited molecular data available (Lax et al. 2019, 2023).

The eponymous genus *Ploeotia* contains 18 described species, most of which were originally characterized only by light microscopy. Consequently, the taxonomy of *Ploeotia* has been repeatedly revised as molecular phylogenetic data have revealed paraphyly within the group. For example, *Ploeotia costata*, which had been transferred from *Serpenomonas costata* in 1988 to *Ploeotia costata* based on ultrastructural data (Farmer and Triemer 1988), was formally transferred back to *Serpenomonas costata* after forming an independent clade within the ploetids in molecular phylogenetic analyses, compared to the phylogenetic placement of the type taxon, *Ploeotia vitrea* (Cavalier-Smith 2016; Lax et al. 2019). To date, only two species examined with molecular data have been demonstrated as true members of the genus *Ploeotia* (*P. vitrea* and *Ploeotia oblonga*), although many of the originally described members of this genus still lack further study beyond light microscopy (Lax et al. 2019). Because several described ploetids still lack molecular and ultrastructural data, continued study of these groups is necessary to further resolve their phylogenetic relationships and elucidate patterns of character evolution among ploetids.

Most well-studied petalomonads and ploetids originate from marine habitats, with comparatively few from freshwater systems examined using molecular data, underscoring freshwater environments as priority targets for discovering and characterizing euglenids, and for reconstructing transitions between marine and freshwater lineages within these groups (Lax and Simpson 2020; Lee et al. 2026). This is particularly true for petalomonads, where most of the described diversity is found

in freshwater environments (e.g., Huber-Pestalozzi 1955). We aimed to identify novel heterotrophic euglenids in freshwater samples from Arizona using cultivation techniques and successfully established three new cultures of heterotrophic euglenids from these environments. These include the ploetids *Hokulea pusilla* comb. nov. and *Ploeotia protuberantia* sp. nov., as well as the novel petalomonaad *Scytomonas flumensis* sp. nov. Here, we formally describe *S. flumensis* sp. nov. and *P. protuberantia* sp. nov. using light microscopy, scanning electron microscopy, and phylogenetic analyses of 18S rDNA sequences. We also transfer *Ploeotia pusilla* to *H. pusilla* comb. nov. based on its morphology and its phylogenetic affiliation with *Hokulea waialeensis* in the phylogeny inferred from 18S rDNA sequences (Kubín et al. 2024; Schroeckh et al. 2003).

2 | Materials and Methods

2.1 | Sampling, Isolation, and Cultivation

S. flumensis and *P. protuberantia* were isolated in 2022 from sediment and water samples from a small creek near Camp Ponderosa (34°16'58.8" N 111°08'34.6" W) in Arizona. *H. pusilla* was collected in 2025 from Salt River (33°25'15.4" N 112°03'53.7" W) in Phoenix, Arizona. In both cases, samples were left in the dark for ~2 weeks to limit growth of photosynthetic organisms. Euglenids were initially identified under bright field microscopy at 400× magnification. The *S. flumensis* and *P. protuberantia* cultures were established by single-cell isolation, in which individually identified cells were transferred into media using a glass-drawn pipette. *H. pusilla* was isolated using enrichment and constant subcultivation. All cells are kept in ventilated cell culture flasks containing freshwater DY-V media (Andersen and Phycological Society of America 2009) kept at 23°C. Cultures were maintained by weekly sub-cultivation in a 1:1 ratio of fresh DY-V medium.

2.2 | Differential Interference Contrast Microscopy (DIC)

Light microscopy of all three isolates was performed using a Nikon (Eclipse Ti fluorescence microscope) inverted microscope with differential interference contrast (DIC) microscopy at 1000× magnification. Multiple video recordings of all isolates were captured, and images were extracted from them using VLC v3.20. Twenty-five cells were measured using FIJI (Table S1).

2.3 | Scanning Electron Microscopy (SEM)

We prepared each cell for SEM using the methods described in Ruiz et al. (2026) with minor modifications. Approximately 10 mL of each culture was transferred into a large Petri dish and vapor-fixed with osmium tetroxide for 10 min at room temperature, followed by post-fixation in 1% osmium tetroxide for 10 min at room temperature. Fixed cells were transferred onto a Millipore membrane filter and washed once in distilled water. Fixed cells were dehydrated using a graded ethanol series (30%, 50%, 70%, 85%, 90%, 95%) followed by three washes in 100% ethanol. Membranes containing fixed cells were critical point dried using a Tousimis Autosamdri 815B critical point dryer. Cells

were sputter-coated with 2 nm Pt/Pd using a Leica EM ACE600 sputter coater. Sputter-coated cell-containing stubs were viewed using a Zeiss XB350 Crossbeam scanning electron microscope at the UBC Bioimaging Facility (University of British Columbia).

2.4 | Single-Cell Amplification, Sequencing, and Assembly

Individual cells of *H. pusilla*, *P. protuberantia*, and *S. flumensis* were manually isolated from cultures and washed 2–4 times in filter-sterilized fresh DY-V media. Individual cells for each species were placed in 0.2 mL PCR strip tubes. The REPLI-g Advanced Single Cell Kit (Qiagen) was used to obtain single-cell amplified genomes (SAG) following the manufacturer's protocol. The resulting SAGs were then quantified using a Qubit BR DNA assay (Invitrogen). For *S. flumensis*, SAGs were sequenced on an Illumina NovaSeq X series instrument with paired end 2×150 bp reads (performed by Psomagen, psomagen.com). Raw reads were quality trimmed using fastp v.0.23.2 (Chen et al. 2018). Trimmed paired reads were used as input for SPAdes version 4.0.0 (Prjibelski et al. 2020) and assembled under single-cell mode with default parameters, which take multiple displacement amplification bias into account (Prjibelski et al. 2020; Spits et al. 2006; Záhonová et al. 2021). Contamination was assessed using Blobtoolkit v4.3.5 (Challis et al. 2020). We used minimap2 v2.1 (Li 2021) under default parameters to determine assembly coverage. Taxonomic affinity was determined by searching each contig against the NCBI non-redundant database using BLASTn v2.16.0+ (Camacho et al. 2009) and against the Uniref90 database using Diamond v2.1.10.164 (Buchfink et al. 2021). Bacterial and other obvious human contaminants we identified were filtered out using Seqkit (Shen et al. 2016). The SAG will be made publicly available in a future manuscript in which more details than 18S will be reported.

2.5 | Phylogenetic Reconstruction

For *S. flumensis*, 18S rDNA sequences were extracted from SAGs using barnapp (<https://github.com/tseemann/barnapp>). For *P. protuberantia* and *H. pusilla*, gDNA from the SAGs for each species were subjected to PCR amplification of the 18S rDNA using OneTaq DNA Polymerase (New England Biolabs). For *P. protuberantia*, euglenid specific primers were used: EzoaF 5' CGCAAATTGCCCAATR 3' and reverse EzoaR 5' GGRGCATCACAGACCTGC 3' (Lax and Simpson 2013) under the following parameters: initial denaturing 94°C for 2 min; then 35× cycles of denaturing at 94°C for 1 min, followed by annealing at 55°C for 1 min, extension at 72°C for 2 min; and a final extension for 5 min. For *H. pusilla*, universal eukaryotic primers were used: EukA 5' AACCTGGTTGATCCTGCCAGT 3' and EukB 5' TGATCCTTCTGCAGGTTACCTAC 3' (Medlin et al. 1988) under the following parameters: initial denaturing at 94°C for 2 min followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 48°C for 35 s, extension at 72°C for 1 min, and the final extension at 72°C for 5 min. The PCR product was run on an agarose gel, gel-purified (Monarch DNA Gel Extraction Kit, New England BioLabs) and then sent to Eurofin Genomics for Nanopore amplicon sequencing (<https://eurofinngenomics.com>). Reads for *H. pusilla*

were cleaned by Eurofin Genomics. Reads for *P. protuberantia* were cleaned by manually stitching the forward and reverse reads. 18S rDNA sequences were deposited on NCBI under the accession numbers PX375662 (*Hokulea pusilla* comb. nov.), PX375663 (*Ploeotia protuberantia* sp. nov.) and PX375664 (*Scytomonas flumensis* sp. nov.). Raw reads generated from SAGs of *S. flumensis* will be deposited in NCBI upon the publication of a subsequent paper that is focused on resolving the backbone of euglenids.

18S rDNA sequences from each species were then aligned with a previously published dataset (Lax and Simpson 2020) using MAFFT E-INS-I version 7.481 (Katoh and Standley 2013). Novel petalomonad and environmental 18S sequences from Lee et al. (2026) were added to the alignment using the—add function implemented on the MAFFT web server (https://mafft.cbrc.jp/alignment/server/add_sequences.html). The resulting alignment was manually corrected and trimmed using the Geneious Prime 2024.0.4 alignment masking algorithm. A Maximum-likelihood (ML) tree of the final alignment of 267 taxa with 1143 positions was estimated with IQTree2 under the GTR+I+F+G4 model with 1,000 non-parametric bootstrap replicates. Untrimmed and trimmed alignments are included in the [Supporting Information](#) of this article.

3 | Results and Discussion

3.1 | *Hokulea pusilla* comb. nov. Is Conspecific With *Ploeotia pusilla* (formally *Ploeotia obliqua*) and Closely Resembles the Sister Species, *Hokulea waialensis*

In our molecular phylogenetic analysis of euglenids inferred from 18S rDNA sequences (Figure 1), *H. pusilla* branched sister to *H. waialensis* (83% bootstrap support) within a larger clade containing both *Decastava* and *Lentomonas*, consistent with the tree topology recovered in our previous study (Ruiz et al. 2026). Both *Hokulea* species form a clade with two environmental sequences, BF-A1-1-3a and BF-A1-1-5a (Ruiz et al. 2026). The pairwise identity between *H. pusilla* and *H. waialensis* is 95.5%.

H. pusilla comb. nov. strongly resembles the previously described ploetid *Ploeotia pusilla* (syn., *Ploeotia obliqua*) in cell size, cell shape and cytoskeletal organization as observed with light microscopy; both isolates are relatively small compared to other ploetids (Figure 2A–D) (Duangjan et al. 2017; Kubín et al. 2024; Lee et al. 2005; Ruiz et al. 2026; Schroeckh et al. 2003). Like *P. pusilla*, *H. pusilla* cells are colorless, rigid and have a slight asymmetrical ovoid shape with a slightly pointed posterior end, reminiscent of a lemon. *H. pusilla* cells have a length of $11.35 \pm 1.21 \mu\text{m}$ and a width of $6.49 \pm 1.07 \mu\text{m}$ (Figure 2A–D). The two flagella of *H. pusilla* originate from the conspicuous flagellar pocket and differ in thickness and length, with the posterior flagellum slightly thicker and considerably longer ($26.33 \pm 4.36 \mu\text{m}$ long; $2.32\times$ cell length) than the anterior flagellum ($6.57 \pm 1.00 \mu\text{m}$ long; $0.58\times$ cell length) (AF, PF and FP, Figure 2A–D,F, Figure 3C). Like other ploetids, the anterior flagellum of *H. pusilla* exhibits a near-constant whipping or sweeping motion in front of the cell, whereas the posterior flagellum extends far beyond the cell and is used for

gliding. A feeding apparatus was not observed with light microscopy in *H. pusilla*, unlike in previous descriptions of *P. pusilla* (Kubín et al. 2024; Lee et al. 2005; Schroeckh et al. 2003); however, this is likely due to the small size of these cells and the resolution of the microscope available.

H. pusilla exhibits differences in cell shape from the type species, *H. waialensis*, when observed with both light microscopy and SEM (Ruiz et al. 2026). While *H. waialensis* has an oblong cell shape, *H. pusilla* is more lemon-shaped, with a distinct tapered end that seems to be characteristic for this species (Figure 2A–D; Figure 3A–C,E). *H. pusilla* cells are slightly dorsoventrally flattened, with some but not all cells appearing concave on the ventral side of the cell (Figure 3B), unlike *H. waialensis*, which, while being slightly dorsoventrally flattened, does not contain a clear depression on the ventral side in any cell observed in our previous study. The cell surface and pellicle organization of *H. pusilla* is nearly identical to that of *H. waialensis*, where the surface of *H. pusilla* is covered in 10 proteinaceous pellicle strips (arrowheads; Figure 3A,D) that are helically arranged, flattened and contain strip junctions that appear even shallower than those of *H. waialensis*. A glycocalyx can be observed covering the cell surface, particularly at the anterior end near the feeding apparatus and flagella (G, Figure 3B,D). Glycocalyx has been observed in other lineages of euglenids, including the type species *Hokulea waialensis*, the flexible phagotroph *Neometanema parovale*, and several osmotrophic species (Leander et al. 2017; Lee and Simpson 2014a; Ruiz et al. 2026).

The surface of both flagella is covered in a dense fibrous matrix likely representing a combination of ordered mastigonemes beneath a glycocalyx, which is present across the entire cell surface (Figure 3C,D). This appears similar to the mastigonemes, tomentum and glycocalyx associated with the flagella near the vestibulum described for *H. waialensis* in Ruiz et al. (2026), Figure 2D. The feeding apparatus of *H. pusilla* also shares several similarities with *H. waialensis* (FA, Figure 3D). It contains hair-like filaments on the exterior surface of the feeding apparatus at the anterior tip of the cell and is continuous with a ventral pellicle strip bordering the vestibular opening (FA, Figure 3B,D). The feeding apparatus of *H. pusilla* appears flush with the anterior end of the cell without extending outwards as dramatically as in *H. waialensis*. The clear similarities and differences in ultrastructure as observed with SEM are consistent with *H. pusilla* and *H. waialensis* forming sister taxa in our phylogenetic analyses of 18S rDNA sequences.

Because there are no transmission electron micrographs (TEM) of any *Hokulea* species, characterizing the ultrastructure of either *H. pusilla* or *H. waialensis* using TEM would help clarify the organization and placement of the feeding apparatus within the cell as well as the structure and arrangement of the pellicle.

3.1.1 | Remarks

P. pusilla and *H. pusilla* are indistinguishable from one another and *H. pusilla* forms the sister lineage to *H. waialensis*, which is well supported with the morphological evidence presented in

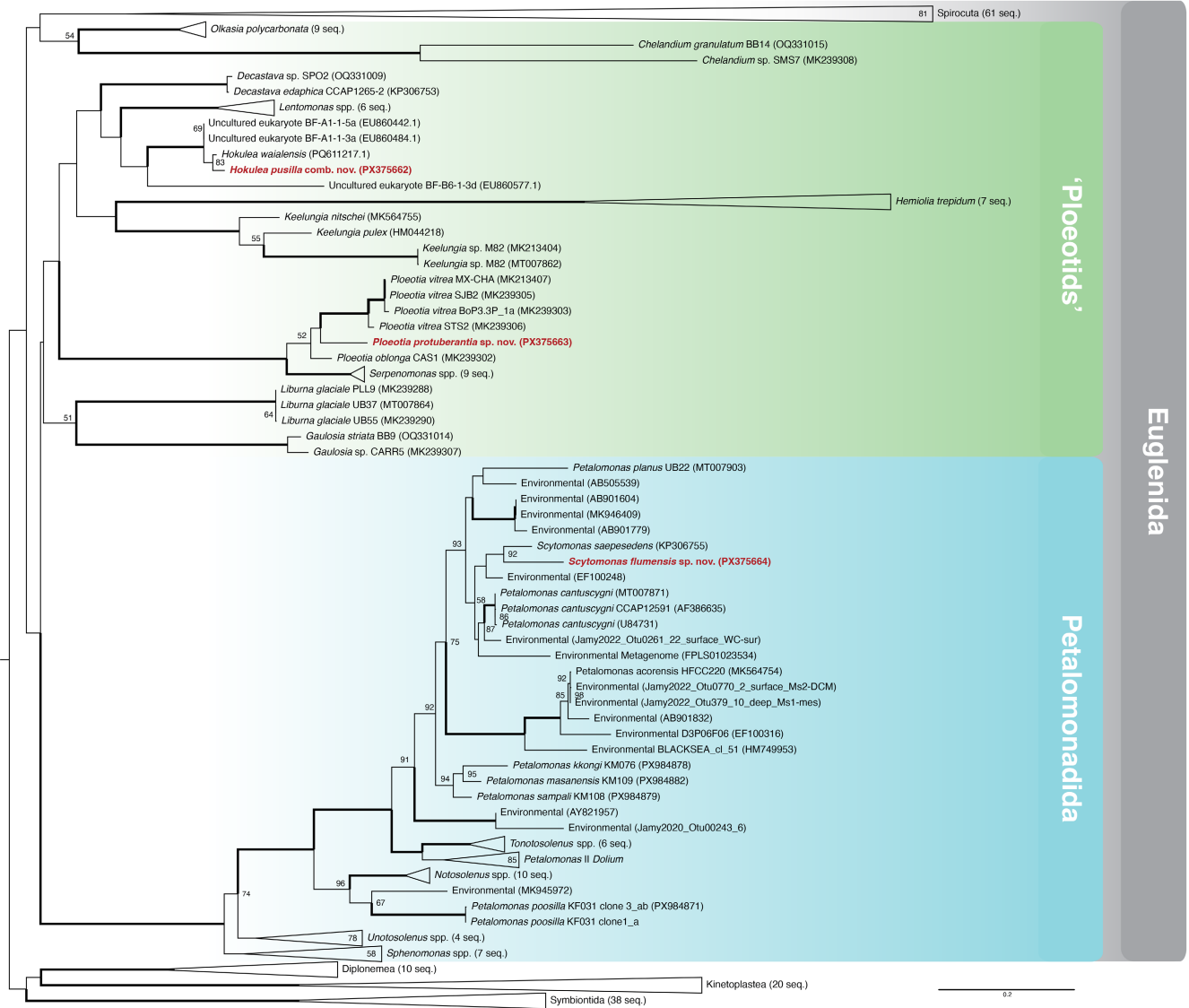


FIGURE 1 | Maximum-likelihood molecular phylogenetic analysis of euglenids inferred using 18S rDNA under the GTR + F + I + G4 DNA substitution model using 1000 non-parametric bootstrap replicates. *Hokulea pusilla* comb. nov., *Ploeotia protuberantia* sp. nov., and *Scytomonas flumensis* sp. nov. sequences are shown in bold. Major groups of euglenids are indicated and the tree is rooted using kinetoplastids, diplomonads, and symbionts as an outgroup. Thickened branches indicate 100% bootstrap support and bootstrap values below 50% are not shown.

this study. As such, we regard *P. pusilla* (previously *P. obliqua*) as a junior synonym (basonym) of *H. pusilla* and transfer it to *Hokulea* based on its placement in phylogenies inferred from 18S rDNA sequences.

3.2 | *Ploeotia protuberantia* sp. nov. Is Closely Related to *P. vitrea* and *P. oblonga*

In our molecular phylogenetic analysis of euglenids inferred from the 18S rDNA sequences, *P. protuberantia* was recovered as a member of *Ploeotia*, branching sister to *P. vitrea* with moderate to low bootstrap support (48%) (Figure 1). *P. protuberantia* was recovered as the sister to *P. oblonga* in some analyses, but in all cases, its placement was recovered within *Ploeotia*. The pairwise identity between *P. protuberantia* and *P. vitrea* (MK213407) and between *P. protuberantia* and *P. oblonga* (MK239306) was 68% and 67.7%.

P. protuberantia cells are rigid, oblong and appear colorless with a rounded posterior end, $21.27 \pm 1.78 \mu\text{m}$ long and $12.42 \pm 1.82 \mu\text{m}$ wide (Figure 2E–H) when observed using DIC light microscopy. Typical of ploeotids, *P. protuberantia* has two flagella originating from the flagellar pocket that differ in thickness and length, with the posterior flagellum being slightly thicker and significantly longer ($37.58 \pm 6.60 \mu\text{m}$ long; $1.77 \times$ cell length) than the anterior flagellum ($8.86 \pm 1.88 \mu\text{m}$ long; $0.42 \times$ cell length) (AF, PF; Figure 2F). The motility of *P. protuberantia* is consistent with that of other ploeotids, where the posterior flagellum is used for gliding while the anterior flagellum whips in front of the cell. *P. protuberantia* contains a hook-shaped feeding apparatus extending nearly the entire length of the cell that closely resembles the feeding apparatus of other ploeotids, namely *Serpenomonas costata*, *Ploeotia vitrea* and *P. oblonga* (FA; Figure 2E). This feeding apparatus contains two rods with a cap at the very distal end near the cell surface, lying adjacent to a ventral pellicle strip (FA; Figure 2E; Figure 4A,B). The pellicle of *P. protuberantia* cells consists of 10

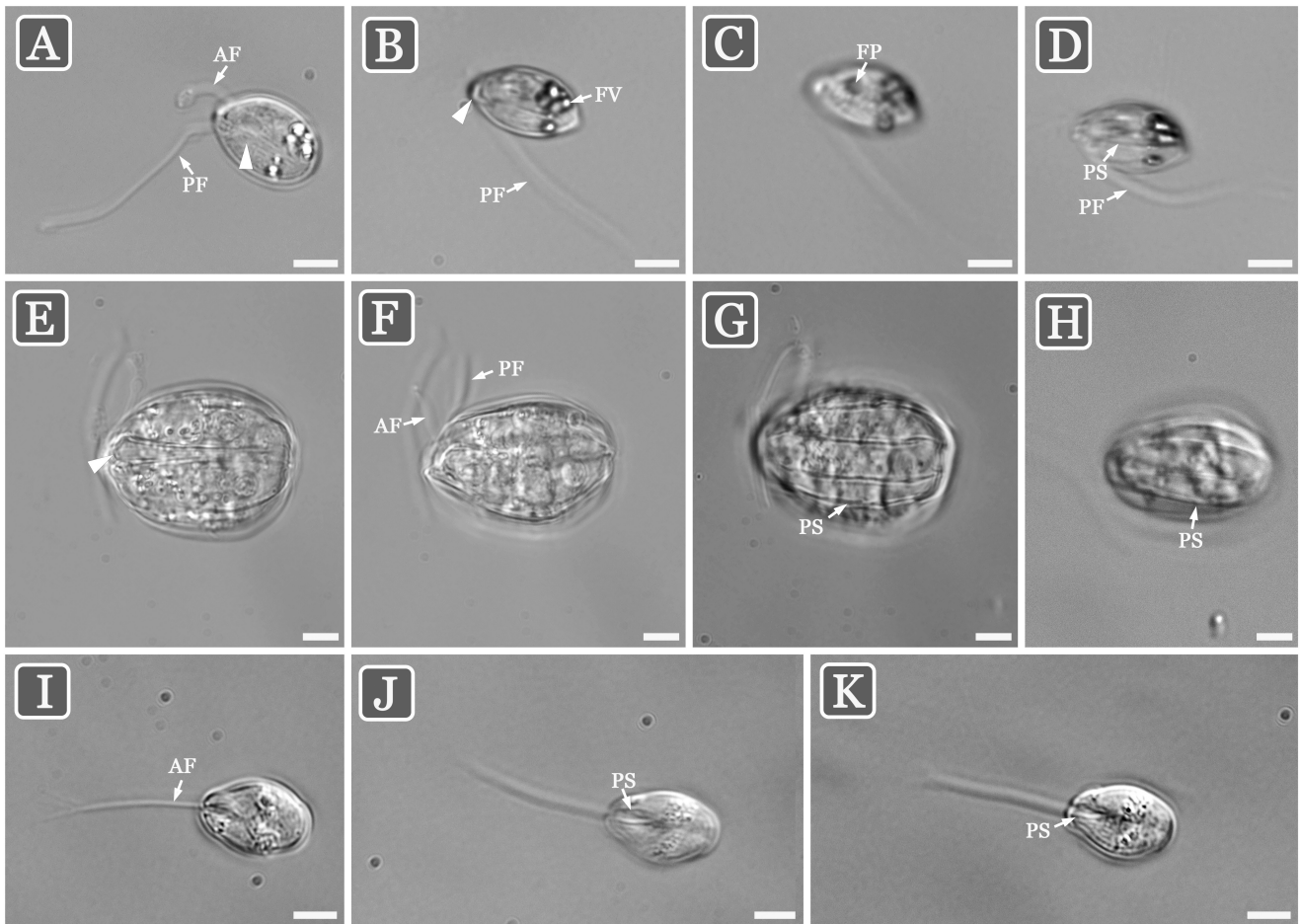


FIGURE 2 | Differential Interference Contrast (DIC) microscopy of *Hokulea pusilla* comb. nov. (A–D), *Ploetia protuberantia* sp. nov. (E–H), and *Scytomonas flumensis* sp. nov. (I–K). (A–D) DIC micrographs of *H. pusilla* showing the anterior (AF) and posterior (PF) flagella, the feeding apparatus (arrowheads), food vacuoles and the flagellar pocket (FP). Proteinaceous pellicle strips (PS) can be seen in (D). (E–H) DIC micrographs of *P. protuberantia* showing the feeding apparatus (arrowhead), the anterior (AF) and posterior (PF) flagella (E–F) and proteinaceous pellicle strips (PS) (G–H). (I–K) DIC micrographs of *S. flumensis* showing the anterior (emergent) flagellum (AF) and proteinaceous pellicle strips (PS). All scale bars = 5 µm.

distinct, longitudinally arranged pellicle strips that are equal in width and form double-edged keels that project outwards, visible with both light microscopy and SEM (PS, Figure 2G–H; arrowheads, Figure 4A,C). Similar to *P. vitrea*, the central ventral pellicle strip is slightly narrower than the remaining strips, although this difference in width is less pronounced in *P. protuberantia*.

There are several key morphological differences between *P. protuberantia* and its close relatives, *P. vitrea* and *P. oblonga*, particularly in the organization of the pellicle. Although both *P. protuberantia* and *P. vitrea* have 10 equally spaced pellicle strips that form double-edged keels at their junctions (Lax et al. 2019), the strip length and how they converge at the posterior end of the cell differs. Unlike in *P. vitrea* and *P. oblonga*, where alternating pellicle strips are slightly shorter than the adjacent strips so the longer ones meet to form a five-pointed star at the posterior end of the cell, in *P. protuberantia*, most of the pellicle strips are the same length, forming an 8–10 pointed star at the posterior end (arrowheads; Figure 4C). *P. oblonga* further differs in having heterogeneously spaced pellicle strips, with every second strip markedly narrower than its neighbor, in contrast to the uniformly spaced strips in *P. protuberantia* and *P. vitrea* (Lax et al. 2019).

A particularly interesting feature of *P. protuberantia* is a consistent and distinct layer of small granules that cover the cell surface (Figure 4). These granules, also present in *P. vitrea* and *P. oblonga* when observed with SEM in Lax et al. (2019), are less than 100 nm in size and appear to be contained within thin web-like fibers (M; Figure 4D). The structure and composition of these granules are unknown for any of these three species in the absence of TEM and warrant further investigation. Regardless, these granules may be a clade-defining trait for *Ploetia* as they have been observed in all confirmed members of this genus and are not present on the surface of any other euglenids.

3.2.1 | Remarks

Considering the close yet distinct morphological traits among *P. protuberantia*, *P. vitrea*, and *P. oblonga*, along with the strong support for *P. protuberantia*'s placement within *Ploetia* in our phylogenies inferred from 18S rDNA sequences, we propose that *P. protuberantia* sp. nov. should be recognized as a novel species of *Ploetia*.

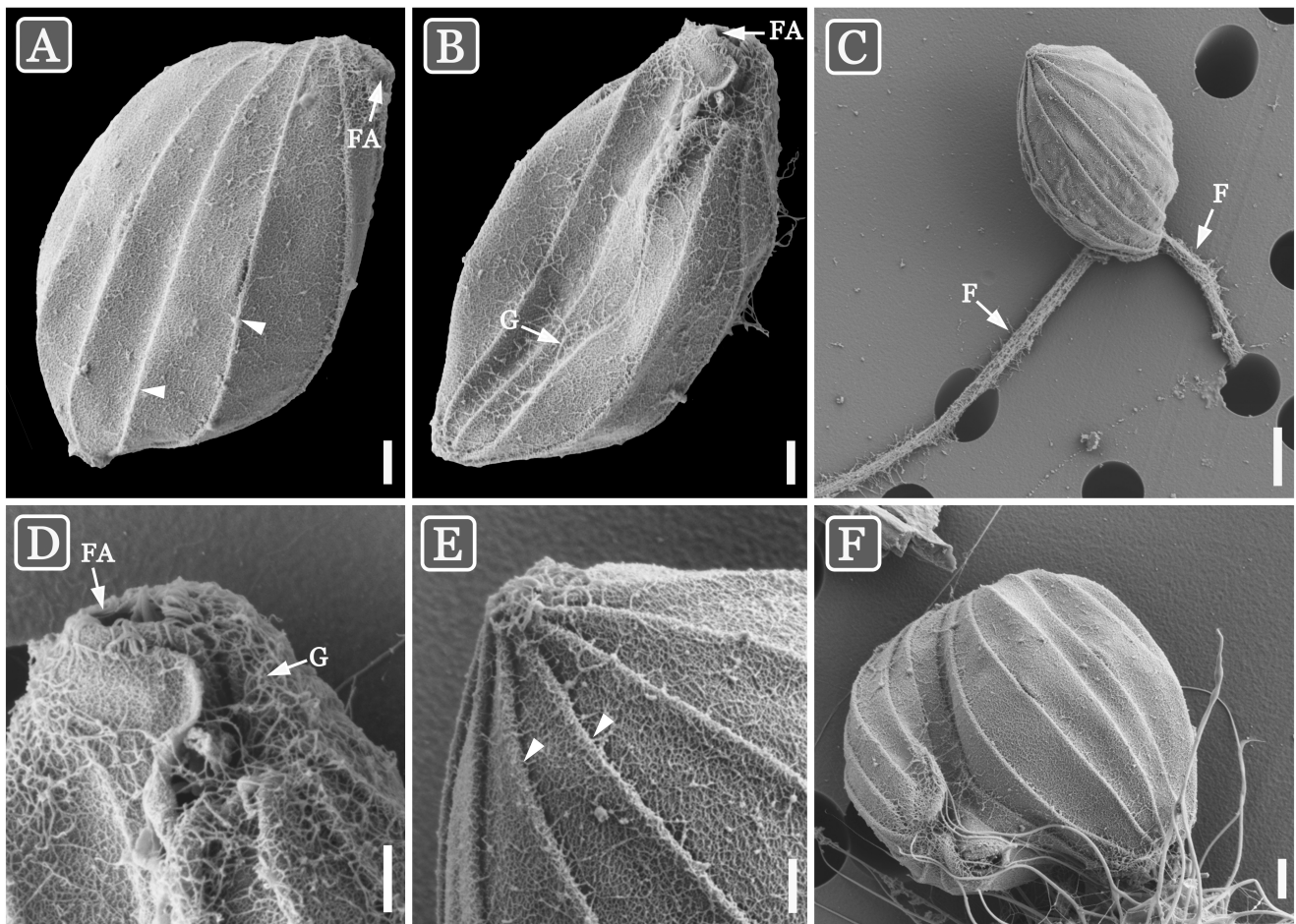


FIGURE 3 | Scanning Electron Micrographs (SEM) of *Hokulea pusilla* comb. nov. (A, B) Dorsal view (A) and ventral view (B) of *H. pusilla* showing the feeding apparatus (FA) and numerous proteinaceous pellicle strips (arrowheads). A glycocalyx can be seen covering the cell surface, particularly on the ventral side (G). (C) Low magnification SEM of *H. pusilla* showing the dorsal side of the cell. Two flagella (F) can be seen. Scale bars A–C = 1 μ m. (D) High magnification view of the feeding apparatus (FA) surrounded by thick glycocalyx (G). (E) High magnification view of the posterior end of *H. pusilla* showing numerous pellicle strip junctions. Scale bars D–E = 500 nm. (F) Low magnification view of *H. pusilla* dividing. This cell is shown next to another protist from the same culture. Scale bar = 1 μ m.

3.3 | *Scytomonas flumensis* sp. nov. Is a Novel Freshwater Petalomonad From Arizona That Is Sister to *Scytomonas saepesedens*

In our molecular phylogenetic analysis of 18S rDNA sequences, *Scytomonas flumensis* sp. nov. was recovered as the sister to *Sytomonas saepesedens* within the *Petalomonas* I, *Scytomonas* clade (Lee et al. 2026) with moderate to high bootstrap support (92%) (Figure 1). The pairwise identity between the 18S rDNA sequences of *S. flumensis* and *S. saepesedens* is notably low for sister species (79%). Although these species are currently sister species in 18S rDNA phylogenies, the low pairwise identity suggests that they may not be true immediate sister species, and future sampling of freshwater petalomonads may uncover hidden diversity within this group.

Cells of *S. flumensis* are small ($9.72 \pm 1.82 \mu\text{m}$ long and $5.82 \pm 1.34 \mu\text{m}$ wide), colorless and have a characteristic oval shape with a slight taper at the anterior end of the cell (Figure 2I–K, Figure 5A,B). These cells possess one long anterior flagellum on which they glide forwards, measuring $11.27 \pm 3.26 \mu\text{m}$ long ($1.16 \times$ cell length). (AF; Figure 2I, Figure 5A,B). This flagellum

is thick in diameter when viewed with SEM and appears to be covered in fine flagellar hairs (Figure 5A–C). *S. flumensis* cells have 5 pellicle strips that are equal in width and are evenly distributed on either side of the cell (arrowheads; Figure 5). These pellicle strips form a distinct depression on the dorsal side of the cell, between two raised pellicle strip junctions, resulting from invagination of the central pellicle strip from the adjacent ridge (arrowheads; Figure 5). The pellicle strip junctions on either side of this depression are prominent and visible under light microscopy (PS; Figure 2J,K). The pellicle strips converge at the posterior end of the cell, at a junction that extends to the dorsal side (arrowheads; Figure 5D). A ventral protrusion (VP; Figure 5C) is present at the anterior end of the pellicle strip that runs adjacent to the flagellar canal, consistent with other ventral protrusions observed in many other petalomonads, including *Tonotosolenus* spp., *Petalomonas kkongi*, *Petalomonas poosilla*, *Petalomonas larseni*, *Scytomonas saepesedens*, among others (Cavalier-Smith et al. 2016, Figure 1B,D; Lee et al. 2026).

Although *S. flumensis* closely resembles *S. saepesedens* in the general organization of the pellicle and its appearance under light microscopy, there are several key morphological differences

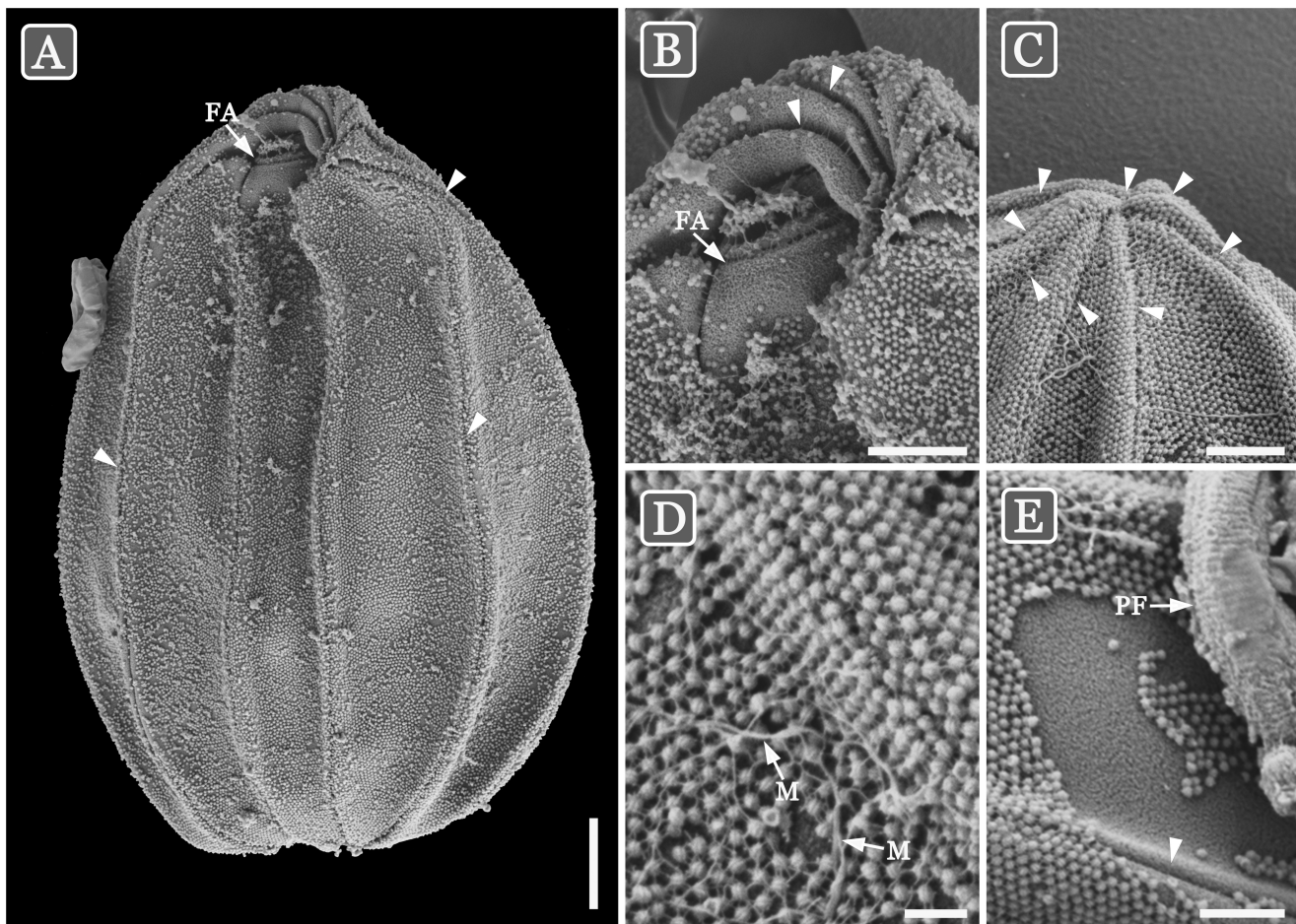


FIGURE 4 | Scanning Electron Micrographs (SEM) of *Ploeotia protuberantia* sp. nov. (A) Low magnification SEM of the ventral side of *P. protuberantia* showing the feeding apparatus (FA) and numerous proteinaceous pellicle strips (arrowheads). The cell surface is covered in a layer of small granules. Scale bar = 2.5 μm . (B) High magnification SEM of the anterior region of the cell showing the feeding apparatus (FA). Pellicle strips converge near the feeding apparatus (arrowheads). (C) High magnification view showing converging pellicle strips (arrowheads) at the posterior end of the cell. Scale bars B–C = 1 μm . (D) High magnification SEM showing the small granules covering the cell surface. Strands of mucilage (M) are visible, associated with the granules. Scale bar = 250 nm. (E) SEM showing a region of the cell surface where the granules have sloughed off, exposing the pellicle. The posterior flagellum can be seen (PF). Scale bar = 500 nm.

between these two species. Namely, *S. flumensis* possesses a clear dorsal depression that is not observed in *S. saepesedens*, which is more spherical when viewed with SEM (Cavalier-Smith et al. 2016). It is unlikely that this depression is a result of a fixation artifact, as this almost crumpled appearance is consistent with other petalomonads observed in Lee et al. (2026) and was observed in all cells across multiple fixation attempts. *S. flumensis* appears more elongated in the cell body and does not possess a pronounced collar, unlike *S. saepesedens*. *S. flumensis* and *S. saepesedens* were also isolated from drastically different environments; *S. flumensis* was recovered from a freshwater creek in Arizona, USA, whereas *S. saepesedens* was isolated from horse manure in Scotland (Cavalier-Smith et al. 2016).

3.3.1 | Remarks

Given the differences in locality, the morphological differences observed under SEM, and the clearly distinct phylogenetic relationship of *S. flumensis* and *S. saepesedens* as sister taxa in phylogenies

inferred from 18S rDNA sequences, we conclude that our isolate of *S. flumensis* sp. nov. represents a novel member of *Scytomonas*.

4 | Taxonomic Summary

Eukaryota; Discoba; Euglenozoa; Euglenida

Hokulea pusilla Palka, Rodriguez-Ruiz, Lax, Garza, Leander and Wideman comb. nov. Basionym: *Ploeotia obliqua* and *Ploeotia pusilla*.

Transfer from: Stokes (1886) (Basionym: *Anisonema pusillum* A.Stokes, Proc. Amer. Philos. Soc. 23: 562, Figure 5. 1886b) = *Ploeotia obliqua* (G.A.Klebs) Schroeckh et al., Hydrobiol. 493: 158–159, Figs 7n, 8k–n. 2003, syn. nov. $\equiv$ *Entosiphon obliquus* G.A.Klebs, Zeit. Wiss. Zool. 55: 390–391, Table 17: Figs 15a–c. 1892., *Ploeotia pusilla* Kubin et al., Protist 175, no. 4, 2024, syn.

Type habitat. Freshwater oxic sediment.

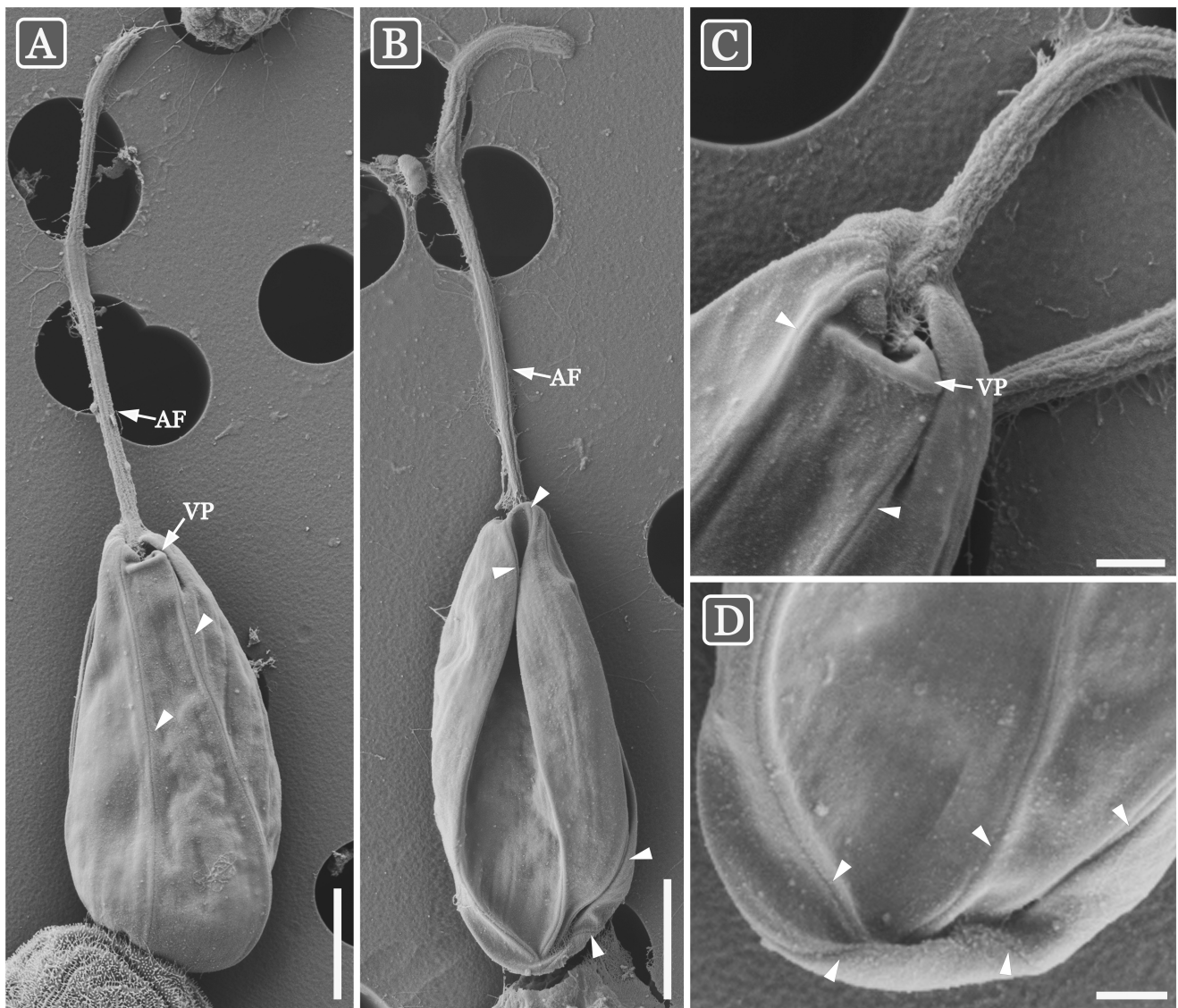


FIGURE 5 | Scanning Electron Micrographs (SEM) of *Scytomonas flumensis* sp. nov. (A–B) Ventral (A) and dorsal (B) views of *S. flumensis* showing the proteinaceous pellicle strips (arrowheads) and long anterior flagellum (AF). The ventral protrusion (VP) is visible in (A). Scale bars = 2.5 μm . (C) High magnification SEM of the ventral side of the anterior end of the cell showing the ventral protrusion (VP). (D) High magnification SEM of the dorsal side of the posterior end of the cell showing five converging proteinaceous pellicle strip junctions (arrowheads). Scale bars C–D = 500 nm.

Type locality. Salt River, Tempe, Arizona, USA (33°25'15.4" N 112°03'53.7" W).

Gene sequence: The 18S rDNA sequence extracted from the single-cell genome amplification product with universal eukaryotic primers and sent out for Nanopore amplicon sequencing to Eurofin Genomics LLC and is deposited under GenBank Accession number PX375662.

Zoobank accession: urn:lsid:zoobank.org:act:BB8EA73C-70A7-49AE-A7F4-575F006D9E02.

Ploeotia protuberantia Palka, Rodriguez-Ruiz, Lax, Garza, Leander, and Wideman spec. nov.

Diagnosis: Oblong, colorless cell with a rounded posterior end. Dorsoventrally flattened, ~21 μm long, ~12 μm wide. The

anterior flagellum and posterior flagellum are ~9 μm (0.42 $\times$ cell length) and ~38 μm (1.77 $\times$ cell length) long, respectively. 8–10 pellicle strips and a conspicuous hook-shaped feeding apparatus are visible with light microscopy. Strip junctions form double-edged keels. Two flagella; the anterior flagellum is shorter and sweeps consistently in front of the cell, while the cell glides on the thicker and longer posterior flagellum.

Type Material: Cells imaged in Figure 2 A–D (iconotype). SEM stub containing cells (hapantotype) is deposited with the Beaty Biodiversity Museum under the accession number MI-PR230.

Type Locality: Camp Ponderosa, Phoenix, Arizona, USA (34°16'58.8" N 111°08'34.6" W).

Etymology: From 'protuberantia' (Latin) meaning protuberations, due to its pellicle strips appearing raised.

Gene sequence: The 18S rDNA sequence extracted from the single-cell genome amplification product with euglenid specific primers and sent out for Nanopore amplicon sequencing to Eurofin Genomics LLC under the GenBank Accession number PX375663.

Zoobank accession: urn:lsid:zoobank.org:act:243FC70D-DEE6-4799-9452-BBFCFDD8B604.

Scytomonas flumensis Palka, Rodriguez-Ruiz, Lax, Garza, Leander, and Wideman spec. nov.

Diagnosis: Elongated, colorless oval cells (~10 µm long, 6 µm wide) with a tapered posterior end that glide using a single anterior-facing flagellum (11.27 ± 3.26 µm long, $1.16 \times$ cell length). Cells contain 5 pellicle strips, with a significant indentation in the pellicle on the dorsal side of the cell that can be viewed with light microscopy.

Type Material: Cells imaged in Figure 2I–K. (iconotype). SEM stub containing cells (hapantotype) is deposited with the Beaty Biodiversity Museum under the accession number MI-PR232.

Type Locality: Camp Ponderosa, Phoenix, Arizona, USA (34°16'58.8" N 111°08'34.6" W).

Etymology: From 'flumen' (Latin) for river, due to the type location in which it was found.

Gene sequence: The 18S rDNA sequence extracted from the single-cell genome amplification product with universal eukaryotic primers and sent out for Nanopore amplicon sequencing to Eurofin genomics LLC and is deposited under GenBank Accession number PX375664.

Zoobank accession: urn:lsid:zoobank.org:act:E9211854-F1E7-4FF6-8D1C-6C9B57D80A37.

Author Contributions

J.G.W. conceived and guided the study. A.R.-R. and Y.G. collected the organisms, obtained the molecular data, and performed the light microscopy within the context of A.R.-R.'s graduate research. M.V.P. conducted the electron microscopy. A.R.-R., M.V.P., and G.L. performed molecular phylogenetic analyses. M.V.P. designed the figures. A.R.-R. and M.V.P. wrote the first draft of the manuscript. M.V.P., A.R.-R., G.L., B.S.L., and J.G.W. edited subsequent iterations of the manuscript. J.G.W. and B.S.L. funded the research.

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Data Availability Statement

The data that supports the findings of this study are available in the [Supporting Information](#) of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Supporting Information. **Table S1:** Cell measurements.