A novel free-living endomyxan flagellate *Viscidocauda repens* gen. nov., sp. nov.

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Endomyxa comprises a diverse group of protists, including free-living amoebae and parasites, that infect various hosts. In this study, we report a new free-living amoeboflagellate, *Viscidocauda repens* gen. nov., sp. nov., isolated from seawater near Hachijojima Island, Japan. *V. repens* is a gliding bacterivorous biflagellate and occasionally extends pseudopodia from its posterior end. Molecular phylogenetic analysis based on small subunit ribosomal RNA gene sequences places *V. repens* as sister lineage to a clade comprising the endomyxan Ascetosporea and Gromiidea. Ultrastructural observations revealed that *V. repens* has four microtubular roots (R1–R4), but lacks vp2, a unique microtubular band widely distributed among cercozoan flagellates. Based on its morphology, ultrastructure, and phylogenetic position, we propose *V. repens* as a new genus and species within Endomyxa.

Keywords: Cercozoa, Endomyxa, flagellate, flagellar apparatus, molecular phylogeny, SSU rRNA, ultrastructure

INTRODUCTION

The rhizarian subphylum Endomyxa comprises a diverse group of protists, including free-living amoebae (Gromiidea and Vampyrellida) and intracellular parasites (Phytomyxea and Ascetosporea) (Bass et al. 2009a; Hartikainen et al. 2014; Hess et al. 2012; Hittorf et al. 2020). Gromiidea is a group of marine filose and reticulose amoebae, including naked net-like amoebae (*Filoreta*) and giant testate amoebae (*Gromia*) (Bass et al. 2009a). Vampyrellida is a group of filose amoebae that alternate between motile amoeboid and immobile cyst stages throughout their lifecycle. They feed on various organisms, including bacteria, algae, and fungi via engulfment or by piercing cell walls (Hess et al. 2012; Hess and Suthaus 2022). Phytomyxea are intracellular parasites that infect plants, diatoms, oomycetes, and brown algae. Some phytomyxean parasites, such as *Plasmodiophora brassicae* and *Spongospora subterranean*, have notable economic impacts, such as those causing clubroot disease in brassicas and powdery scab disease in potatoes (Balendres et al. 2016; Dixon 2009). Ascetosporea includes intracellular parasites of marine invertebrates, such as *Haplosporidium nelson*, which is known to cause MSX disease in eastern oysters (Burreson and Ford 2004).

Unlike the rhizarian subphylum Filosa, free-living flagellates have not previously been reported in Endomyxa. Endomyxan protists lack flagella or produce them exclusively at specific life stages, such as zoospores (Bass et al. 2009a). Environmental DNA surveys targeting small subunit ribosomal RNA (SSU rRNA) have revealed several deep-branching clades within the Endomyxa (Bass and Cavalier-Smith 2004; Bass et al. 2009a). Despite the phylogenetic significance of these environmental clades, only Novel Clade 8 has been associated with a known group, Vampyrellida (Bass et al. 2009a). This highlights the need for further research to explore the diversity and evolutionary relationships within the Endomyxa.

In the present study, we present a new amoeboflagellate that occupies a basal position within Endomyxa. We describe the organism *Viscidocauda repens* gen. nov., sp. nov., as a new genus and species based on molecular phylogenetic analysis and morphological and ultrastructural observations. Additionally, we explore the diversity and evolution of Endomyxa by integrating morphological and molecular perspectives.

MATERIALS AND METHODS

Sample collection and culture establishment

Seawater samples were collected from a depth of 5 m in the Pacific

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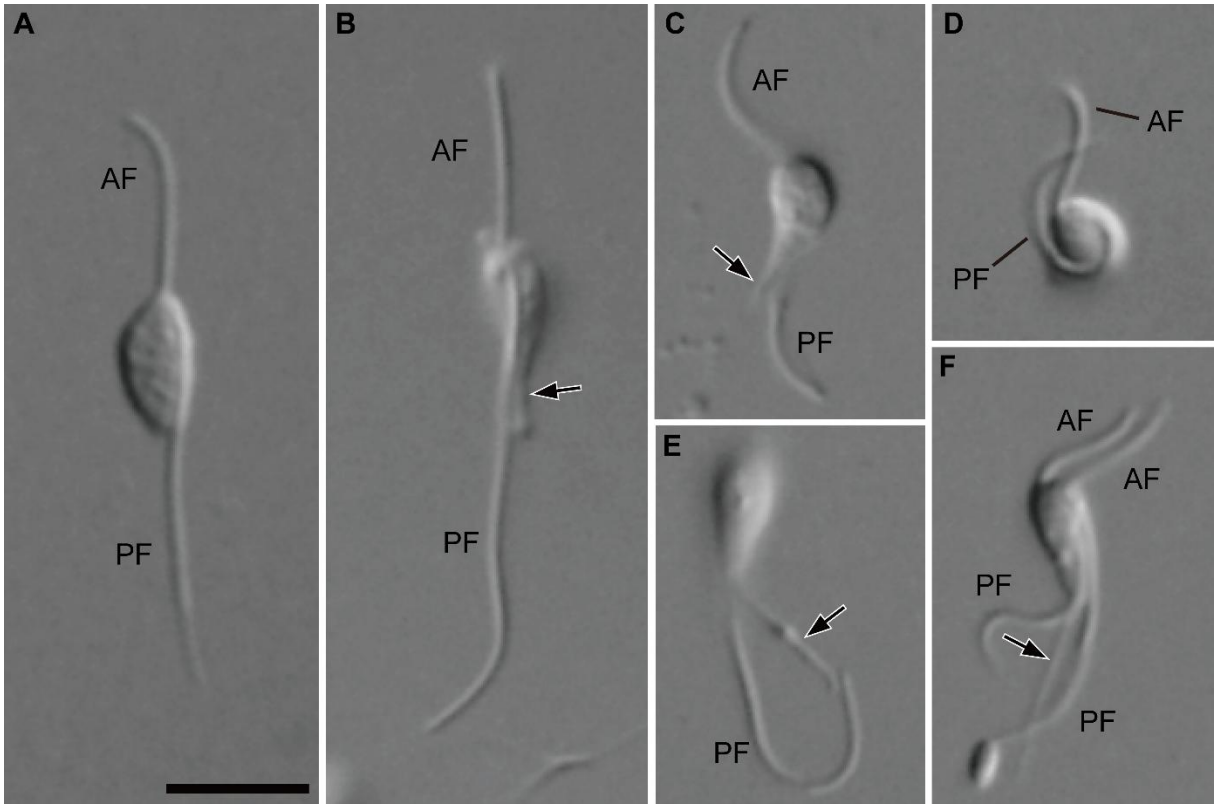

Figure 1 Differential interference contrast (DIC) micrographs of *Viscidocauda repens* gen. nov., sp. nov. **A.** Lateral view of a cell. **B–E.** Ventral view of cells showing pseudopodia. **F.** A cell with duplicated flagella. Arrows indicate pseudopodia. Scale bar = 5 μm.

Ocean near Hachijojima Island (33.00°N, 140.00°E) on September 6, 2012, during cruise KT-12-23 of the R/V Tansei-maru. The sample was filtered through a 20 μm filter and then concentrated using a 2 μm filter. The filtered sample was incubated in ESM medium (Kasai et al. 2009) for two weeks at room temperature. A clonal culture of *Viscidocauda repens* gen. nov., sp. nov., was established from the sample through micropipetting isolation and maintained in ESM medium at 20°C under continuous dark conditions until December 2020.

Light microscopy

Cells of *V. repens* were placed in a glass-bottomed dish and observed under an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP71 CCD camera (Olympus, Tokyo, Japan).

Electron microscopy

Specimens of *V. repens* were prepared as previously described (Shiratori and Ishida 2023). Ultrathin sections were prepared using a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria), double-stained with 2% (w/v) uranyl acetate and lead citrate, and observed using a Hitachi H-7650 electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Veleta TEM CCD camera (Olympus).

DNA extraction and sequencing

Total DNA extraction, PCR for the small subunit ribosomal RNA (SSU rRNA) gene, and SSU rRNA gene sequencing of *V. repens* were performed as previously described (Shiratori and Ishida 2024). Total DNA from the *V. repens* was extracted from the cell pellets collected by centrifugation using a DNeasy Plant Mini Kit (Qiagen Science) according to the manufacturer's instructions. The small subunit ribosomal RNA (SSU rRNA) gene fragment of the strain was amplified using PCR using primers 18F and 18R (Yabuki et al., 2010). Amplified DNA fragments were purified after gel electrophoresis using a QIAquick Gel Extraction Kit (Qiagen Science) and then cloned into the pGEM T-easy vector (Promega). The inserted DNA fragments were sequenced using a 3130 Genetic Analyzer (Applied Biosystems) and BigDye Terminator v3.1 cycle sequencing Kit (Applied Biosystems). The SSU rRNA gene sequences of *V. repens* were deposited in GenBank under accession code LC847298.

Molecular phylogenetic analysis

For the molecular phylogenetic analysis, 85 SSU rRNA gene sequences were obtained from the NCBI database. The SSU rRNA gene sequence of *V. repens* was added to the dataset and aligned using MAFFT v7.526 (Katoh and Standley 2013). Ambiguously aligned regions were manually deleted using SeaView version

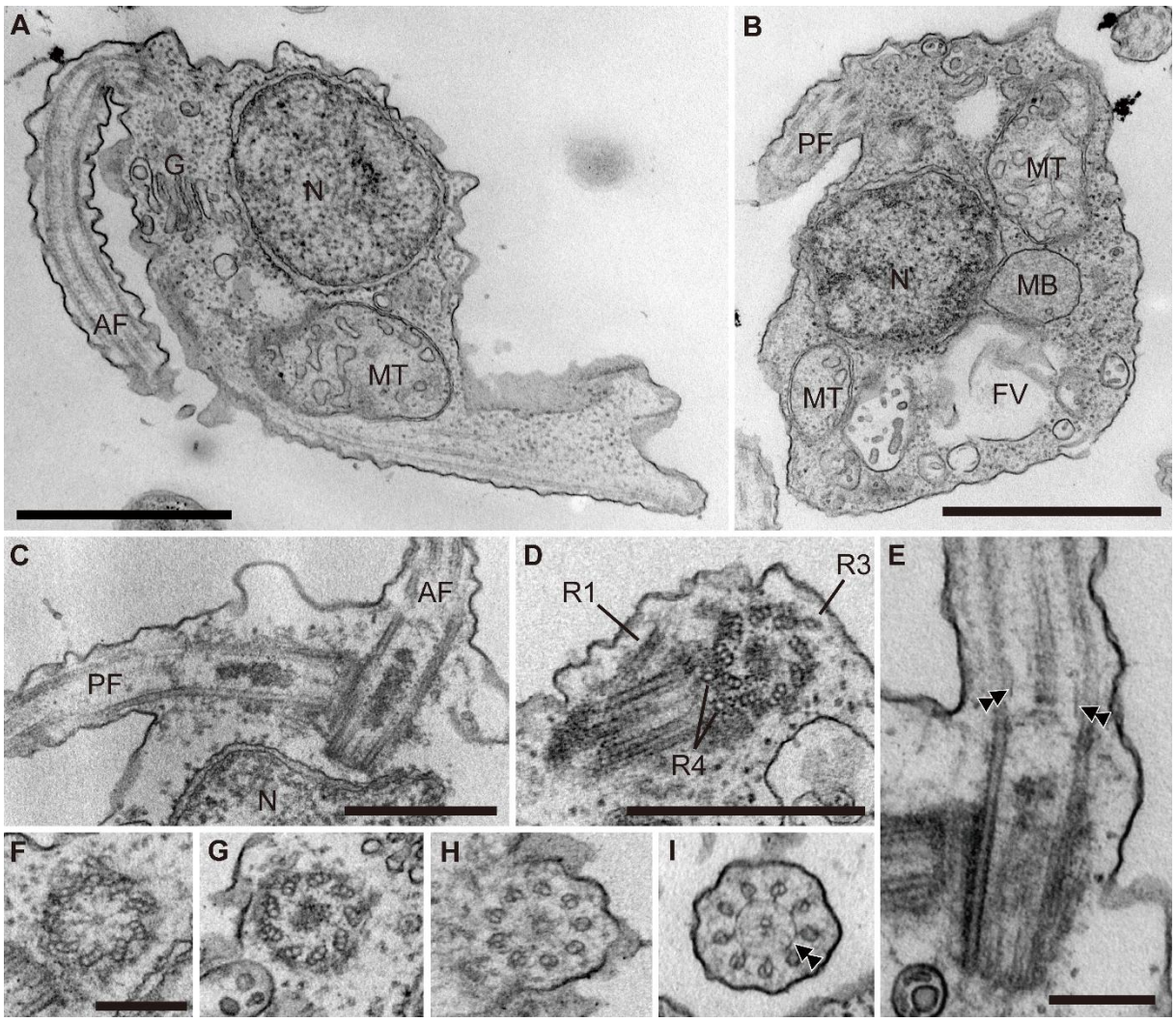

Figure 2 Transmission electron micrographs of *Viscidocauda repens* gen. nov., sp. nov. **A, B.** General cell image. **C.** Longitudinal section of two basal bodies. **D.** Cross section of the anterior basal body. **E.** Longitudinal section of the anterior basal body. **F.** Cross section of the proximal part of the basal body. **G.** Cross section of the middle part of the basal body. **H.** Cross section of the transitional region. **I.** Cross section of the proximal end of the flagellum. Double arrowheads indicate ring structures in transitional regions. AF, anterior flagellum; G, Golgi apparatus; N, nucleus; MB, microbody; MT, mitochondrion. PF, posterior flagellum. Scale bars: **A, B** = 1 μm, **C, D** = 500 nm. **E–I** = 200 nm

5.0.5 (Gouy et al. 2010), resulting in a final dataset comprising 1711 nucleotide positions. The maximum likelihood (ML) tree was searched using IQ-Tree v.1.6.12 (Nguyen et al. 2015) with the TIM3+F+R4 model. Branch supports were obtained using an ultrafast bootstrap (BP, 1000 replicates) with an IQ-Tree (Hoang et al. 2018). Bayesian analysis was carried out using MrBayes v.3.2.6 (Ronquist et al. 2012) with the GTR+Γ model. One cold and three heated Markov chain Monte Carlo simulations with default chain temperatures were run for 8×10^6 generations, sampling the lnL values and trees at 100-generation intervals. Convergence was assessed using the average standard deviation of the split frequencies, and the first 25% of all generations in each analysis were discarded as 'burn-in.' Bayesian posterior probabilities (BPP) and branch lengths were calculated from the remaining trees.

RESULTS

Cells of *Viscidocauda repens* gen. nov., sp. nov., are metabolic and oval or obovoid shaped, 2.8–5 (3.8 ± 0.7, n = 20) μm in length and 1.4–2.4 (1.9 ± 0.3, n = 20) μm in width (Fig. 1). The cells possess two heterodynamic flagella inserted subapically from the ventral side (Figs 1A–C, D). The anterior flagellum, which is approximately 1–2 times its cell length, exhibits dynamic beating. The posterior flagellum, approximately 2–4 times its cell length, extends posteriorly along the cell body (Figs 1A–C, D). Lobose or filose pseudopodia were occasionally observed at the posterior

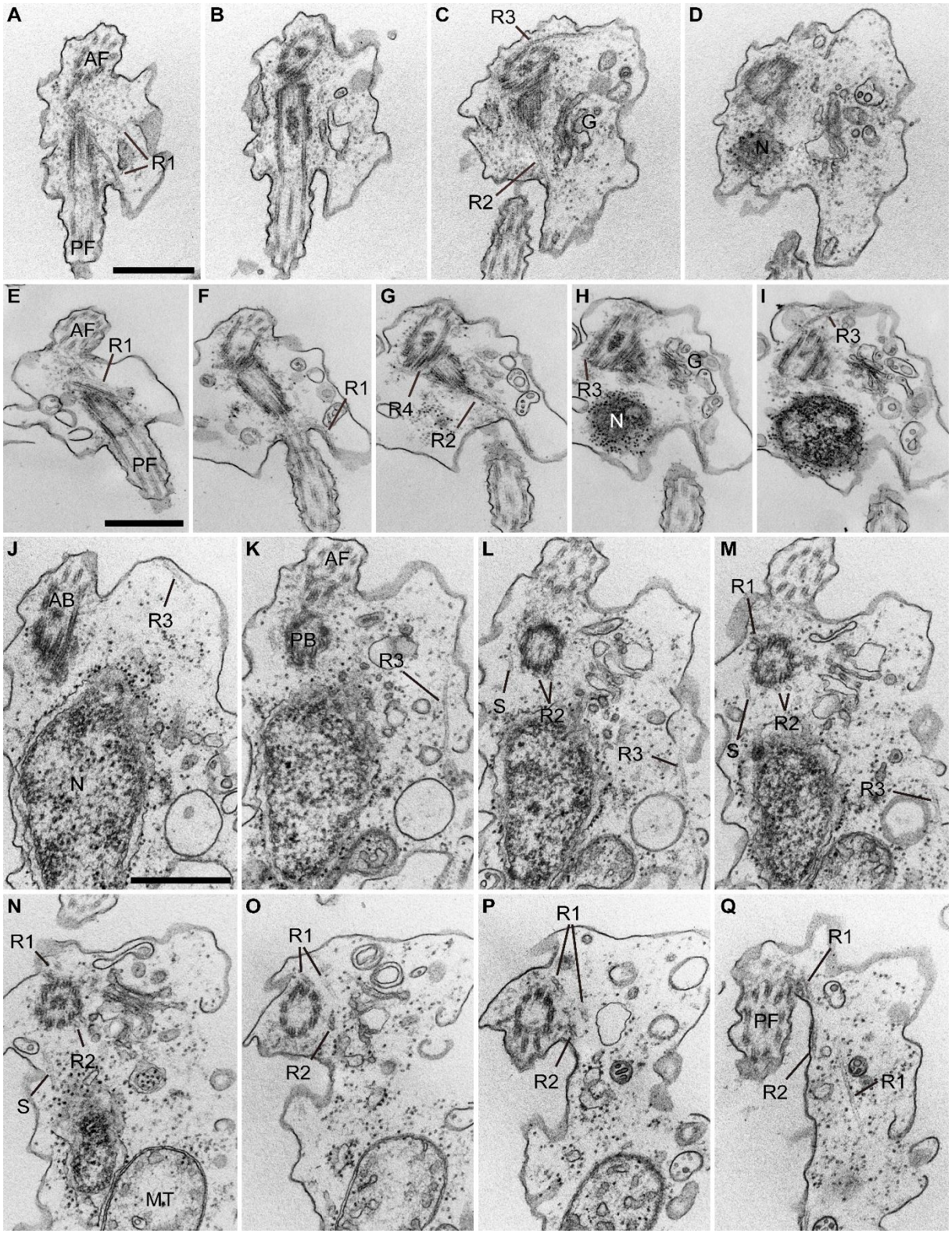

Figure 3 Transmission electron micrographs of *Viscidocauda repens* gen. nov., sp. nov. **A–D.** Selected serial sections viewed from the anterior side of a cell. **E–I.** Selected serial sections viewed from right anterior side of a cell. **J–Q.** Selected serial sections viewed from the ventral side of a cell. AB; anterior basal body, AF, anterior flagellum; N, nucleus; MT, mitochondrion. PB; posterior basal body, PF, posterior flagellum. Scale bars = 500 nm.

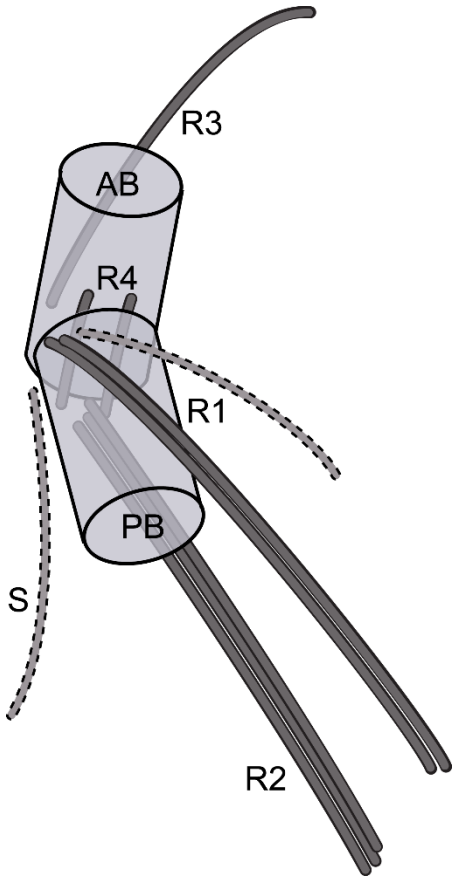

Figure 4 Illustration of the flagellar apparatus of *Viscidocauda repens* gen. nov., sp. nov. AB, anterior basal body; PB, posterior basal body.

ends of cells (Figs 1B, C, E, and F). The cells glide slowly on the substrate. During gliding, the posterior pseudopodia and posterior flagellum make contact with the substrate, while the anterior half of the cell typically remains elevated above the substrate. The anterior half of the gliding cells display a side-to-side vibrating motion synchronized with the beat of the anterior flagellum. No cysts or non-flagellated stages were observed.

Ultrastructural observations showed that *V. repens* possesses a nucleus, mitochondria with tubular cristae, a Golgi apparatus, and a microbody (Figs 2A, B). The nucleus is located immediately posterior to the basal body and slightly dorsal to the anterior half of the cell (Fig. 2A). The Golgi apparatus, which is associated with the nuclear envelope, is situated near the basal bodies (Fig. 2A). Two basal bodies containing electron-dense materials are arranged at angles of 90°–110° (Fig. 2C). The flagellar transitional region includes a ring structure (Figs 2E, I). To describe the microtubular roots of *V. repens*, we used the terminology of Yubuki et al. (2013), by assigning posterior basal body to B1 and the anterior basal body to B2. We found some variation in the microtubular roots among individuals, as depicted by the dotted lines in Fig. 4. The R1 root is composed of two microtubules originating from the anterior side of B1 (Figs 2D, 3A, E, and M), and extends posteriorly along the ventral surface of the cells (Figs 3M–Q). We also observed an additional microtubule occasionally emerging near the proximal end of R1, splitting leftward (Figs 3A, O, and Q). The R2 root consists of three microtubules originating from the posterior side of B1 (Figs 3C, G, L–Q). R2 merges with R1 and extends posteriorly along the ventral surface of the cells (Figs 3A–I). R1 and R2 reach the posterior end of the cell and extend into the pseudopodia (Fig. 2A). We occasionally observed that the SR, a singlet microtubule which originates from the right side of B1 (Figs 3L–N), extends posteriorly, terminating in the middle of the cell. The R3 root is a singlet microtubule originating from the dorsal side of B2 (Figs 2D, 3C, and I). The R3 initially extends anteriorly before curving posteriorly along the left dorsal side of the cells (Figs 3J–M). The R4 root, consisting of two short microtubules with a gap, originates from the ventral side of B2 and is situated between B1 and B2 (Figs 2D, 3G).

Molecular phylogenetic analysis of the SSU rRNA gene sequences indicated that *V. repens* is closely related to six environmental sequences obtained from both surface and deep seawater, as well as the gut contents of herbivorous fish (Fig. 5). Six of these sequences showed close affinities with *V. repens*, with differences ranging from 0.3% to 2.6%. *V. repens* and these environmental sequences formed a clade with Ascetosporea, Gromiidea, and the environmental clades Novel Clade Endo-4 and Endo-5, with robust supports (BP = 91%, BPP = 0.9882). Our analysis did not recover the monophyly of Endomyxa; the *V. repens* + Ascetosporea + Gromiidea clade was positioned as a sister to the Filosa + Skiomonadea clade, and the Phytomyxa + Vampyrellida clade was positioned as basal to Cercozoa.

DISCUSSION

Our molecular phylogenetic analysis indicated that *V. repens* branches within an endomyxan subclade, with moderate statistical support. Endomyxa comprises free-living amoebae and parasites that either entirely lack flagella or produce them during specific life stages. Therefore, *V. repens* is the first known endomyxan flagellate that retains its flagella throughout its life history. In contrast, other groups of Cercozoa, Filosa, and Skiomonadea include amoeboflagellates (Bass et al. 2009b; Chantangsi and Leander 2010; Howe et al. 2011). The Glissomonad genus *Neoheteromita* shares several morphological characteristics with *V. repens*. Both are small and metabolic, with the posterior end of the cell adopting an amoeboid form that helps anchor the cell to substrates (Howe et al. 2009). The anterior end exhibits a characteristic flickering motion due to movement of the anterior flagellum (Howe et al. 2009). However, unlike *V. repens*, the well-known glissomonad species, including *Neoheteromita*, are derived from soil and freshwater environments, and no marine species have been reported to date.

The flagellar apparatus of *V. repens* exhibited notable differences compared to the filosan and skiomonadean heterotrophic flagellates. In filosan heterotrophic flagellates, a distinct microtubular band vp2 is commonly observed excluding

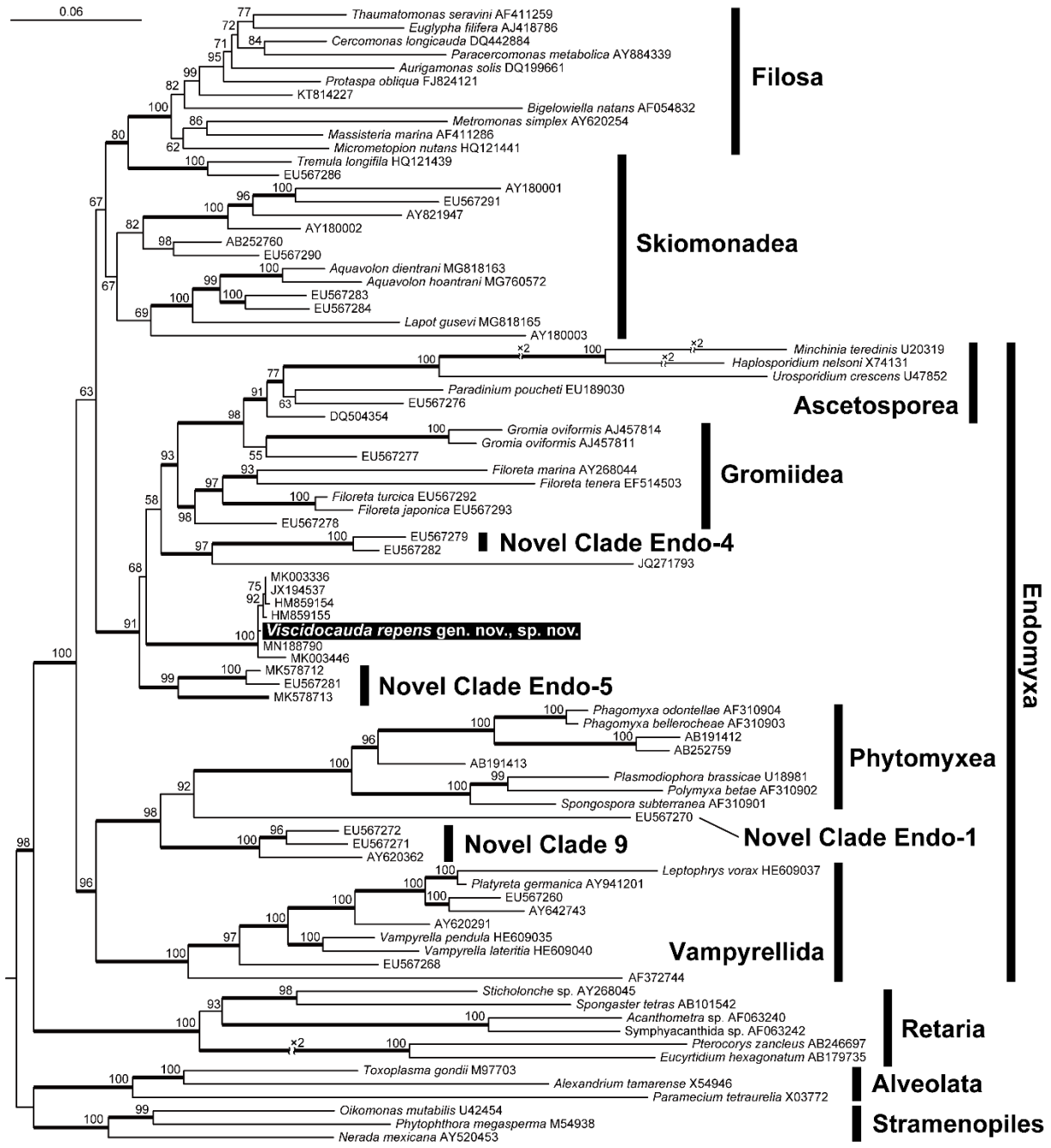

Figure 5 Molecular phylogenetic tree using 1731 nucleotide positions of 83 SSU rRNA gene sequences. Environmental sequences are labeled only with accession numbers. Values at each branch indicate the bootstrap probability (≥50% shown). Bold branches indicate Bayesian posterior probabilities ≥0.95.

those within Thecofilosea (Cavalier-smith 2012; Karpov 2010; Shiratori and Ishida 2020; Shiratori et al. 2014). Vp2 originates from B2 or the associated fibrillar material and extends posteriorly along the ventral surface of the cell, accompanied by the vp1 band, which arises from B1 or nearby fibrillar material. Yubuki et al. (2013) refer to vp1 and vp2 as R2 and R4, respectively, while vp2 also being considered a Filosa-specific microtubular band (Cavalier-Smith 2012). Interestingly, the Skiomonadean flagellate *Aquavolon hoantrani* also possesses a microtubular band, mb2, which appears to be homologous to vp2 (Bass et al. 2018), suggesting that this feature may indicate an ancestral trait within Cercozoa. In contrast, *V. repens* has four microtubular roots, two of which (R1 and R2) emerge from B1 and extend posteriorly along the ventral cell surface. However, no microtubular band similar to that of vp2 was observed. This characteristic of *V. repens* distinguishes it from typical cercozoan heterotrophic flagellates

and is more closely related to other eukaryotic lineages, such as Metamonada, Discoba, malawimonads, Stramenopiles, apusomonds, and CRuMs (Brugerolle et al. 2010; Heiss et al. 2013; O'Kelly et al. 1999; Shiratori et al. 2015; Yabuki et al. 2018; Yubuki et al. 2013). These observations suggest that the flagellar apparatus of *V. repens* might reflect a more ancestral state within Cercozoa.

Our molecular phylogenetic analysis did not recover the monophyly of Endomyxa. Although Endomyxa was originally proposed as a subphylum within Cercozoa (Cavalier-Smith and Chao 2003), its phylogenetic status remains controversial. Some phylogenomic studies have suggested that Endomyxa may be paraphyletic or polyphyletic and can be split into two separate clades: the Ascetosporea + Gromiidea clade and the Vampyrellida + Phytomyxea (Burki et al. 2010; Hiltunen Thorén et al. 2024; Sierra et al. 2013, 2016). However, the other phylogenomic analysis recovered the monophyly of Endomyxa and Cercozoa (Irwin et al. 2019). In future phylogenomic studies, the inclusion of new endomyxan lineages, such as *V. repens*, may help clarify the monophyly of Endomyxa and better define its phylogenetic position within Rhizaria.

We identified six environmental sequences related to *V. repens* originating from various marine samples, including surface and deep seawater, as well as fish gut contents. On the other hand, these environmental sequences displayed low genetic diversity, suggesting that *Viscidocauda* may include few species but widely distributed in marine environments.

The combination of gliding motility and pseudopodia is common in Filosa and has also been observed in Skiomonadean flagellates (Bass et al. 2018; Howe et al. 2011). The discovery of *V. repens* supports the idea that gliding amoeboflagellates represent an ancestral state of Cercozoa and suggests that the endomyxan subclade comprising Ascetosporea, Gromiidea, and *Viscidocauda* is an ancestrally flagellate. Further taxonomic studies on other unidentified Endomyxa lineages based on both morphological and molecular approaches are required to elucidate the evolution and diversity of this enigmatic group.

TAXONOMIC TREATMENT

Phylum Cercozoa
Subphylum Endomyxa
Viscidocauda gen. nov.
Diagnosis: Marine gliding bacterivorous biflagellates with filose or lobose pseudopodia from the posterior end of the cell. The anterior flagellum is shorter than the posterior flagellum. Mitochondrial cristae tubular. A transitional plate and ring structure are present in the flagellar transitional region. Refractile bodies are absent. Microbody present. Extrusomes absent.
Type species: *Viscidocauda repens*
Etymology: The genus name "Viscidocauda" derived from the Latin adjective "Viscidus" (sticky) and the Latin noun "Cauda" (tail), referring to the posterior pseudopodia of the cell. *Viscidocauda* is considered feminine.
Zoobank Registration: urn:lsid:zoobank.org:act:C33440E7-2C13-4018-97D1-6983862F5C5C

Viscidocauda repens sp. nov.
Diagnosis: Cells oval or obovoid, 2.8–5 μm in length and 1.4–2.4 μm in width. The anterior flagellum is approximately 1–2 times the cell length, and the posterior flagellum is approximately 2–4 times the cell length. Two basal bodies at angles of 90°–110 °Cells attach to the surface at their posterior end and posterior flagellum and show slow gliding movements. Cysts absent.
Hapantotype: One EM block (TNS AL-66007tba) was deposited in the herbarium of the National Museum of Nature and Science (TNS) in Tsukuba, Japan.
Paratype: One EM block (TNS AL-66007tbb) was deposited in the TNS. These cells were derived from the same sample as that of the hapantotype.
DNA sequence: LC847298
Type locality: Seawater samples were collected at a depth of 5 m from the Pacific Ocean near the Hachijojima Islands (33.00°N, 140.00°E), Tokyo, Japan.
Collection date: September 6, 2012
Etymology: Specific epithet "*repens*" derived from the Latin present participle of *repo* (creep), referring to the gliding movement of the species.
Zoobank Registration: urn:lsid:zoobank.org:act:95915658-0437-4A2D-962A-27E47F5BE564.

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