The amoebae of *Idionectes vortex* (Cutosea, Amoebozoa): Motility, cytoskeleton architecture and extracellular scales

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Abstract

The Cutosea represent a deep-branching lineage within the phylum Amoebozoa that is still relatively poorly explored. Currently, there are four cutosean representatives known – the monotypic genera *Armaparvus*, *Idionectes*, *Sapocribrum*, and *Squamamoeba* – with marked genetic distances. *Idionectes vortex* is the deepest-branching species and differs markedly from the other Cutosea in ecology, life history, and most importantly, in its ability to form a flagellated swarmer with an exceptional swimming mechanism. As far as we know, the other Cutosea lack flagella and rather represent small, marine amoebae with a characteristic cell coat. The present paper focuses on the amoeboid life history stage of the algivorous amoeboflagellate *Idionectes vortex* to provide data for a first in-depth comparison with other Cutosea and to document structural specialties. The amoeboid stage of *Idionectes* is mainly associated with the specific feeding process, that is, the interaction with algal prey cells and phagocytosis of protoplast material. Yet, the present data from time-lapse microscopy, cytochemical stainings, and electron microscopy demonstrate clear similarities with the other cutosean species concerning amoeboid locomotion and cell coat ultrastructure. Furthermore, *Idionectes* amoebae exhibit a well-developed microtubular cytoskeleton, and an unusual basal apparatus that seems to undergo marked changes during the life history of this exceptional amoebozoan.

KEYWORDS
actomyosin, Amoebozoa, basal body, cell coat, cilia, Cutosea, filopodia, flagella, kinetosome, pseudopodia

THE Amoebozoa Lühe, 1913 is a morphologically diverse phylum of protists that not only comprises the stereotypic, naked amoebae such as the popular *Amoeba proteus*, but also testate amoebae, cellular and plasmodial slime molds, and a number of flagellate life forms (Cavalier-Smith et al., 2015, 2016; Kang et al., 2017). The flagellate taxa are of special evolutionary interest, as flagella are relatively rare within the Amoebozoa, but still found in several major lineages of the “Evosea” clade (Kang et al., 2017). Many of these flagellate forms are actually amoeboflagellates; that is, they combine amoeboid and flagellate characteristics. Some amoeboflagellates are permanent flagellates with amoeboid tendencies, while others switch between distinct amoeboid and flagellate life history stages. Flagellate taxa occur
in the Variosea (e.g., *Phalansterium*, *Cavostelium*, and *Multicilia*), Eumycetozoa (slime molds with flagellate stages), Archamoebae (e.g., *Mastigamoeba*, *Pelomyxa*, and *Rhizomastix*), and Cutosea (*Idionectes*), and interestingly, differ markedly in flagellar structure and motility (Hess et al., 2019; Kang et al., 2017). For example, some Archamoebae (e.g., *Mastigamoeba*) lack outer dynein arms and show reduced flagellar motility (Walker et al., 2001), other Archamoebae (e.g., *Pelomyxa*) have immobile flagella with deviating numbers of axonemal microtubules (Griffin, 1988), the sessile flagellates of the genus *Phalansterium* exhibit fairly stiff but motile flagella for food capture and swarming (Hibberd, 1983; Shmakova et al., 2018), and the multiflagellate predators *Artodiscus* and *Multicilia* have slow-moving flagella used for gliding locomotion (Mikrjukov & Mylnikov, 1998; Ntakou et al., 2019).

The discovery of the parasitoid amoeboflagellate *Idionectes vortex* revealed that flagellate cells are also present in the Cutosea (Hess et al., 2019). Based on our current knowledge, *Idionectes* represents the deepest-branching member of that group and deviates strongly from the other known cutoseans, which are relatively small (< 10 µm), bacterivorous, marine amoebae without any known flagellar stages (Kudryavtsev & Pawlowski, 2013; Lahr et al., 2015; Schuler & Brown, 2019). *Idionectes* is a freshwater amoeboflagellate with a complex life history comprising flagellate swarmers and trophic amoebae. As a “protoplast feeder,” it penetrates the cell walls of filamentous conjugating green algae (*Zygnematophyceae*) and feeds specifically on the algal cell contents. However, the most peculiar feature of *Idionectes* is its unique swimming mechanism that relies on a rotary eukaryotic flagellum and raises some evolutionary questions (Hess et al., 2019).

To better understand the evolution of the Cutosea, further exploration of this yet poorly sampled clade and detailed morphological analyses of its members are desirable. In the original description of *Idionectes vortex*, some typical cutosean characteristics were already evident, for example, the presence of a scaly cell coat. However, due to the past focus on the exceptional swimming mechanism of the swarmer, a detailed microscopic and ultrastructural analysis of the *Idionectes* amoebae is still to be done. The present paper fills this gap with details on the morphology, locomotion, cytoskeleton architecture, and cell coat ultrastructure, and allows for a first in-depth comparison of this extraordinary amoebozoan with its nonflagellate relatives.

**MATERIALS AND METHODS**

**Organisms and cultivation**

All experiments were done with strain UFO.01 of *Idionectes vortex* cultivated as described in Hess et al., 2019. In brief, well-grown cultures of the filamentous conjugating green alga *Zygnema pseudogedeanum* (strain CCAC 0199) were inoculated with *I. vortex* and incubated in the dark or dim light at 15–21°C. After depletion of the algae, the bottom of the culture flasks was full of motile amoebae that were harvested for the experiments.

**Light microscopy and time-lapse photography**

Light microscopy and time-lapse photography were performed with a Zeiss IM35 inverted microscope equipped with the objective lens Plan 100/1.25, differential interference contrast (DIC) optics, a CMOS camera head DFK 72AUC02, and the associated software IC Capture (both The Imaging Source Europe). Time-lapse images were reduced to the green channel, gamma-adjusted with Adobe Photoshop CS4, and finally merged to video files with Adobe Premiere Elements 11 (both Adobe Systems) and VideoPad Video Editor (NCH Software).

**Immunocytochemistry, phalloidin staining, and confocal laser scanning microscopy**

Amoebae were detached from the bottom of culture flasks by shaking, pipetted onto cover slips, and let sediment for ~1 h in a moist chamber. Attached amoebae were then chemically fixed by replacing the fluid with MT buffer (30 mM HEPES, 15 mM KCl, 5 mM EGTA, and 5 mM MgSO4; pH 7) containing 4% formaldehyde and 0.25% glutaraldehyde, and incubated for 10 min at room temperature. Cells were then washed with TBS (150 mM NaCl and 50 mM Tris/HCl; pH 7.5) for 5 min, permeabilized with 0.25% Triton X-100 in TBS for 5 min, blocked with albumin/gelatin (3% bovine serum albumin, 0.1% teleostean gelatin, and 0.05% Triton X-100 in TBS) for 10 min, washed with TBS for 5 min, and incubated with a monoclonal primary antibody produced in mice (clone DM1A against α-tubulin (#T6199) diluted 1:500 or clone 6-11B-1 against acetylated tubulin (#MABT868) diluted 1:400; both Merck) for ~1 h at 37°C. Subsequently, cells were washed with TBS for 5 min (two times), blocked again for 10 min, and incubated with a secondary antimouse antibody produced in goat (IgG DyLight 488 diluted 1:1000; Thermo Fisher Scientific) for ~1 h at...
37°C. After incubation, the cells were washed with TBS for 5 min, stained with phalloidin-Cy3 (diluted 1:80; generously provided by H. Faulstich, Max-Planck-Institut für Zellbiologie) and 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml), washed with TBS again, and mounted on glass slides with a medium containing 85% glycerol, 14% phosphate-buffered saline, and 1% 1,4-diazabicyclo[2.2.2]octane. The cells were finally imaged with a Leica TCS SPE system (SP5) and the Leica LCS software (Leica Microsystems), using appropriate laser lines and filter settings. CLSM image stacks were processed (pseudocolour, Z-projections, merged channels) with the image processing package Fiji (Schindelin et al., 2012) and Adobe Photoshop CS4 (Adobe Systems).

**Scanning electron microscopy**

Amoebae were detached from the substrate by shaking and injected into modified Parducz’ fixative (six parts 2% osmium tetroxide solution and one part saturated mercury chloride solution), incubated for ~5 min at room temperature, collected by centrifugation, then briefly washed with distilled water, and again collected by centrifugation. The cell suspension was then distributed to poly-l-lysine-coated coverslips and let sediment for ~1 h in a moist chamber. The cells were dehydrated with a graded ethanol series (30%–50%–70%–90%–100%, each step 5 min) at room temperature and subjected to critical point drying in a Polaron E3100 critical point drier (Quorum Technologies) using carbon dioxide. Dry samples were then sputter coated with gold in a Sputter Coater 108 Auto (Cressington Scientific Instruments), and imaged with a LEO 430 scanning electron microscope (LEO Electron Microscopy).

**Transmission electron microscopy**

Amoebae were processed for TEM as described for *Idionectes* flagellates in Hess et al., 2019. In brief, amoebae were suspended, fixed with 1.25% glutaraldehyde and 0.5% osmium tetroxide in half-strength Waris-H medium (McFadden & Melkonian, 1986), dehydrated with ethanol, and embedded in EPON with propylene oxide as transitional solvent. The polymerized EPON blocks were sectioned (~60 nm) with a Leica EM UC6 ultramicrotome (Leica Microsystems), stained with uranyl acetate (2%) and lead citrate (Reynolds, 1963), and imaged with a Tecnai-12 transmission electron microscope (FEI Company), a Gatan Orius 832 10.7 megapixel CCD camera, and DigitalMicrograph version 1.83.637 Beta software (Gatan). For TEM whole mounts, suspended amoebae on pioloform-covered copper grids were fixed in osmium tetroxide vapor for 1–2 min, let settle, and air dried after removal of excess fluid.

**RESULTS**

**Morphology, locomotion, and cytoskeleton architecture of amoebae**

The amoeboid cells of *I. vortex* differed clearly from the flagellates as they displayed several conical pseudopodia and a less rugose surface (Figure 1A,B). The amoebae could have various outlines, from isodiametric to elongate, and frequently changed shape during locomotion (Movie S1). If a flagellum was present (before transformation to the swarming flagellate), it emerged from a rugose flagellar pit that was usually closely situated to the nucleus and often surrounded by several asynchronous contractile vacuoles (see also fig. 1c in Hess et al., 2019). The amoebae of *I. vortex* displayed a unique pseudopodial morphology with very thin, needle-like processes emerging from the blunt conical pseudopodia (Figure 1A–C). These processes, previously introduced as “leptopodia” (Hess et al., 2019), take part in the amoeboid locomotion of *I. vortex* as revealed by time-lapse photography (Movie S1). The leptopodia grew from the tips of the conical pseudopodia on the dorsal and anterior sides of the cell, and then moved toward the substrate. After contacting the substrate, they often stopped growing, attached over their entire length, and developed circular adhesion zones at their bases (Figure 1C, 1–3). At the highest resolution, a faint circular margin could be seen in the adhesion zones, probably representing the margin of the scaly surface coat of the cell (Figure 1C inset). The cell body moved over these adhesion zones while forming new leptopodia in the anterior part. The adhesion zones at the posterior end of the locomoting cell became smaller, then detached (sometimes producing very thin, trailing filaments), and were finally absorbed by the cell body (Movie S1). Hence, *I. vortex* performed a walking locomotion involving the continuous generation of dorsofrontal leptopodia that developed into circular, transient adhesion zones on the ventral side of the cell. Chemically fixed amoebae that have been stained with fluorescent phalloidin showed a pronounced concentration of filamentous actin (F-actin) in the leptopodia (including their conical bases) and in the circular adhesion zones (Figure 1D). The signal for phalloidin-positive F-actin was generally found in the cell periphery (a thin actin-rich cortex) and in the hyaline zones of the cells (Figure 2A). As revealed by immunocytochemistry with
antibodies against alpha-tubulin (α-tub). *I. vortex* amoebae also displayed an elaborate microtubular cytoskeleton that was confined to the granular regions of the cell (Figure 2A,B). Irrespective of the presence of a flagellum, the cytoplasmic microtubules originated from a microtubule-organizing center (MTOC) and did not show a noticeable order (Figure 2B). DAPI stainings for DNA showed that the nucleus was not always close to the MTOC, and that there was no strict spatial relationship between these two structures (Figure 2B). The MTOCs and their microtubular rootlets could be visualized with antibodies against acetylated tubulin (ac. tub). Each MTOC consisted of a spiral structure of about 1.5 μm in diameter (yet often appearing as a ring) with numerous radiating microtubular rootlets of approx. 5 μm (Figure 2C–H). The immunocytochemical stainings revealed that the amoebae differed greatly in the stage of flagellar development. In flagellate cells, the flagellum originated from the center of the MTOC spiral (Figure 2C,D), while other cells contained flagellar stubs or only a basal body (Figure 2E). In some instances, no basal body could be detected, while the spiral was still well developed (Figure 2F–H). In these nonflagellate cells, the MTOC was sometimes located well within the cell, and a flagellar pit (typical for the flagellate cells) was not evident. The MTOC in nonflagellate cells did also not show any specific positional relationship to the nucleus.
Ultrastructure of leptopodia, adhesion zones, and the scaly cell coat

TEM whole mounts of detached amoebae revealed that the leptopodia protruded through the scaly cell coat that covered the entire cell body (Figure 3A). The leptopodia were of constant diameter (nontapering) and naked; that is, they were not covered by any visible structures (Figure 3B). This was confirmed by ultrathin sections through the leptopodia, which also revealed the presence of fine, longitudinally oriented filaments (Figure 3C). Attachment sites to external structures (probably corresponding to the adhesion zones observed by light microscopy) were also found, confirming that they are devoid of the scaly cell coat as well (Figure 3D). The scales of the cell coat did not touch the plasma membrane directly, but showed a more or less constant distance of about 38 nm (31–52 nm; n = 20) (Figure 3E,F). Cross sections through the scales showed that they consist of an electron-dense base plate and oblique
margins with an angle of about 120° (110°–130°; n = 12) (Figure 3E). The margins displayed a fine striation parallel to the baseplate (Figure 3F). Glancing sections through the cell coat revealed that the scales are boat-shaped; that is, they have broadly fusiform outlines (Figure 3G). The base plates of the scales were about 150 × 70 nm (n = 21) and exhibited two marginal rows of 6–8 electron-translucent puncta that might be pores (Figure 3G,H).
DISCUSSION

Despite the fact that the Cutosea represent a relatively deep branch in the amoebozoan phylogeny, this group is still poorly sampled and understood from the point of the cellular organization (Schuler & Brown, 2019). The discovery of *I. vortex*, a parasitoid freshwater cutosean with a flagellate life history stage and an exceptional mode of flagellar motility, raises some questions concerning the evolution of cellular traits in the Cutosea. The published structural data about the other three known cutosean representatives, all of which inhabit marine ecosystems and seem to be pure amoebae without known flagellate stages (Kudryavtsev & Pawlowski, 2013; Lahr et al., 2015; Schuler & Brown, 2019), allow for some interesting comparisons. Here, it was demonstrated that amoebae of *I. vortex* perform a walking locomotion on surfaces that involves the generation of well-defined, circular adhesion zones (Figure 4A, B). While information on cell motility of the very small cutoseans *Sapocriburum chincoteaguense* and *Armaparvus languardus* is yet limited (Lahr et al., 2015; Schuler & Brown, 2019), amoeboid locomotion has been well described for *Squamamoeba japonica* (Kudryavtsev & Pawlowski, 2013). This species does not form leptopodia, but it shows a pattern of cell locomotion that is similar to that found in *I. vortex*. *Sq. japonica* was reported to form small, mammiliform subpseudopodia in the anterior and lateral region of the cell (see fig. 1B and G in Kudryavtsev & Pawlowski, 2013), which then develop into “rounded spots” on the ventral side, the latter of which may correspond to the adhesion zones in *I. vortex*. The subpseudopodia of *Sq. japonica* resemble the conical pseudopodia of *I. vortex* as both structures contain cytoplasmic processes that protrude through breaks in the scaly cell coat. In *I. vortex*, these processes develop into the long, needle-like leptopodia, while they are relatively small and inconspicuous in *Sq. japonica* (see fig. 1G and 2D in Kudryavtsev & Pawlowski, 2013). As shown by the phalloidin staining and supported by the microfilaments seen in the ultrathin sections, the leptopodia of *I. vortex* are based on F-actin, which is in line with our general knowledge of thread-like pseudopodial structures, especially filopodia (Gupton & Gertler, 2007; Leijnse et al., 2015; Mattila & Lappalainen, 2008). The processes of *Sq. japonica* also contain some electron-dense, potentially filamentous material, which is clearly devoid of ribosomes (see fig. 2D in Kudryavtsev & Pawlowski, 2013). It is, therefore, likely that the pseudopodia of *Sq. japonica* and *I. vortex* represent two different versions of the same cellular structure in these larger cutoseans (Figure 4C). Long and thin pseudopodia protruding through the scaly cell coat were also documented for the minute *Sapocriburum chincoteaguense* (see fig. 1A–C and 2F in Lahr et al., 2015), but there is no indication that they would develop into broader adhesive structures. It might well be that the mode of locomotion of the very small cutoseans *S. chincoteaguense* and *Armaparvus languardus* deviates from that of larger cutoseans as a side effect of size reduction. Given the internal phylogeny of cutosean taxa with *Idionectes* at its base (Hess et al., 2019), and the similarities found in *I. vortex* and *Sq. japonica*, the common cutosean ancestor might have been a walking amoeba (with the genetic ability to form a flagellum).

The exact function of the relatively long leptopodia of *I. vortex* remains unknown, but the situation in *Sq. japonica* demonstrates that leptopodia are not essential for the walking locomotion. These taxa differ, however, strongly in their feeding ecology. As many other small and mid-sized amoebozoans, *Sq. japonica* consumes bacteria that are continuously grazed from surfaces during amoeboid locomotion (Kudryavtsev & Pawlowski, 2013). In contrast, *I. vortex* has a much more specialized life history with a flagellate stage for dispersal, and it consumes just a selection of filamentous zygnematophycean green algae in a parasitoid-like manner (Hess et al., 2019). The leptopodia might also be used for probing (algal) surfaces more efficiently, and to increase the chance of detecting suitable host cells in a structurally complex microhabitat such as algal mats. Many predatory protists feeding on other eukaryotes, for example, heliozoa and certain vamyprellids, rely on long and slender cell extensions increasing the chance of prey contact (e.g. Hess, 2017; Hülsmann, 1993; Patterson & Hausmann, 1981; Suzuki et al., 1980).

The amoebae of *I. vortex* contain an elaborate microtubular cytoskeleton that is clearly separate from the F-actin-rich domains. The relatively dense microtubular cytoskeleton without a noticeable order resembles the situation in other phylogenetically diverse amoebozoans, for example, species of the genera *Acanthamoeba* (Preston, 1985), *Physarum* (Uyeda & Furuya, 1985), *Dictyostelium*, *Vannella*, *Flabellula*, *Cochliopodium* (Tekle & Williams, 2016), and *Stratorugosa* (Melton et al., 2019). Irrespective of the presence of a flagellum, the *I. vortex* amoebae contained a prominent MTOC that was similar to that known from the flagellates (see fig. 3h in Hess et al., 2019). However, the MTOCs observed in the amoebae displayed different stages of flagellar development (full-grown flagella and stubs), and in some cases, there was no visible basal body. Prior to cell division, many eukaryotes duplicate the basal bodies, which later act as nucleation site (“centrosomes”) for the spindle microtubules during mitosis (Joukov & De Nicolo, 2019; Yubuki & Leander, 2013). Thus, the absence of the basal body is surprising, but might relate to the aberrant overall organization of the basal apparatus in *I. vortex*, that is, a rotating flagellum and a unique flagellar anchorage that resembles a rotary joint (Hess et al., 2019). The present data indicate that the MTOC and flagellar anchorage undergo marked changes during the life history of *I. vortex* that deserve further in-depth examination in the future. Furthermore, it will be very interesting to analyze the microtubular cytoskeleton and the structure of potential MTOCs in other cutosean representatives that are not known to produce flagella. In the current phylogeny of the
Cutosea, *I. vortex* occupies the deepest-branching position and a comparison of the MTOCs in different species might provide some insights into the evolution of the rotary flagellum in *I. vortex*.

The extracellular scales of *I. vortex* have a distinct ultrastructure, with a fusiform outline and well-developed oblique margins (Figure 4D). They do not display a noticeable arrangement, and the scaly cell coat covers the entire cell except the flagellum, leptopodia, and adhesion zones. This indicates that the extracellular envelope is flexible and dynamic and has the ability to transiently open for pseudopodial protrusions and attachment zones. Similar scaly cell coats have been documented for the other members of the Cutosea and are considered a common and characteristic trait of this amoebozoan lineage (discussed in Cavalier-Smith et al., 2016; Schuler & Brown, 2019). The different cutosean species show some variation in scale ultrastructure (shape, size, and appendages), but the overall morphology,
arrangement, and topology of the scales in these amoebae are rather uniform, including those of *I. vortex* (Figure 4E). All known cutosean cell coats show a marked distance to the plasma membrane and lack visible attachment structures in ultrathin sections (Hess et al., 2019; Kudryavtsev & Pawlowski, 2013; Lahr et al., 2015; Schuler & Brown, 2019), raising some questions about how these coats move and reassemble during amoeboid locomotion. Yet, results from scanning electron microscopy suggest that there might be amorphous material between the scales, potentially an organic matrix that is not well preserved or easily contrasted during the preparation for transmission electron microscopy (only in *Sq. japonica* some “material of medium electron density” was reported; Kudryavtsev & Pawlowski, 2013). This material is localized between the scales of all known cutoseans (see inset fig. 1d in Hess et al., 2019 and fig. 3B, D, F in Schuler & Brown, 2019) and deserves some future analyses.

All in all, the morphology, motility, and ultrastructure of *I. vortex* amoebae aligns very well with the data from other Cutosea. The marked differences between cutosean taxa might relate to major differences in their ecology and life history. Further exploration of yet unknown Cutosea and detailed analyses of their cellular traits will eventually draw a clearer picture of how these characters evolved within the group, and how certain species adapted to their specific environments.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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