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Ultrastructure of the gills ciliary epithelium of Limnoperna fortunei (Dunker 1857), the invasive golden mussel

Erico Tadeu Fraga Freitas (ericotadeu@ufmg.br)
Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas
Amanda Maria Siqueira Moreira
Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas
Rayan Silva de Paula
Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas
Gabriela Rabelo Andrade
Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas
Marcela David de Carvalho
Companhia Energética de Minas Gerais SA (CEMIG)
Paulo Santos Assis
Universidade Federal de Ouro Preto (UFOP)
Antônio Valadão Cardoso
Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas
Erika Cristina Jorge
Universidade Federal de Minas Gerais (UFMG)

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Abstract

Background *Limnoperna fortunei* is a bivalve mollusk originally from southern Asia that invaded South America in the 1990's. Its high efficiency in pumping and filtering water and the capacity to promote strong adhesion to different substrates allowed the adaptation of this invasive species, associated with several environmental and economic damages. A deep understanding of the biology and ecology aspects of *L. fortunei* is necessary to outline effective strategies to manage its invasion. Mollusk gills are important structures responsible for several biological functions including breathing and feeding. In this work, we characterized the ultrastructure of *L. fortunei* gills and its ciliary epithelium using transmission and scanning electron microscopy. This is the first report of the *L. fortunei* gills ciliary epithelial cells visualized with high resolution and detailed morphology.

Results The analysis showed a highly organized and large amount of ciliary structures (frontal cilia, laterofrontal cilia, and lateral cilia) on the entire length of the branchial epithelium. Mitochondria, smooth endoplasmic reticulum and glycogen granules were abundantly found in the epithelial cells of the gills, suggesting that all this energetic apparatus could be related to the morpho-functional structure of the cilia. Vesicles possibly containing mucus could also be observed in these cells, suggesting that they might be related to *L. fortunei* mechanism of selection and/or rejection of captured particles suspended in water.

Conclusions Our data suggest the mechanism used by this mollusk for particles capture and selection, which could contribute to a better understanding of important aspects of invasion and decide on more efficient and economic strategies of population control.

Background

The biological invasion carried out by alien animals and plants is one of the threats to biodiversity in aquatic ecosystems. Among the known invasive species, mollusks of the class Bivalvia are responsible for causing both environmental and economic damages [1]. The golden mussel (*Limnoperna fortunei* (Dunker 1857)) and *Corbicula fluminea* (Müller 1774) are representatives of these invading bivalves in South America [2, 3]. *Limnoperna fortunei* is a bivalve belonging to the family Mytilidae (subclass Pteriomorphia and order Mytiloida) and native to Southeast Asia (including China and South Korea) [4]. The arrival of this invasive mollusk in South America occurred in early 1990's, possibly transported by ballast waters from cargo ships coming from Asia due to the increase in business routes between the countries of these two continents [5].

Limnoperna fortunei can inhabit waters with a wide range of temperatures and salinity and handle long-term periods of air exposure [6, 7]. The full morphological knowledge of *L. fortunei* structures is important to highlight the understanding of bioinvasion aspects. Some recent studies focused on the functional surface of the foot [8] and of the shell microstructure of *L. fortunei* [9]. As a prolific filter feeder, the clearance rates of the golden mussel are among the highest reported for suspension feeding bivalves,

including other invasive species such as *Dreissena polymorpha* (Pallas 1771), *Dreissena bugensis* (Andrusov 1897), and *C. fluminea* [10]. Besides capturing suspended particles, bivalves are also able to select them and mucociliary analysis of bivalve gills, including *L. fortunei*, might serve as a tool to measure environmental quality as these mollusks filter and accumulate particles that can be transferred and distributed to other trophic levels [11]. Bivalves can function as bioindicators, such as blue mussels that are often used as sentinels in coastal pollution monitoring [12]. Indeed, this was already evaluated in studies involving the accumulation and dynamics of microplastics [13] and herbicides, such as glyphosate [14, 15].

Morphologically, *L. fortunei* is a headless mollusk that has a single foot enclosing the visceral mass, two pairs of gills (ctenidia) and gonochoric with external fertilization. Each individual has two valves surrounding the body composed of calcium carbonate [16] and its polymorphs aragonite and amorphous calcium carbonate [9]. Gills are in direct contact with the environment, suggesting their importance in xenobiotics biotransformation, antioxidant response, signal transduction, innate immune response, and osmoregulation [17, 18]. Furthermore, a giant virus belonging to the Marseilleviridae family was recently found in *L. fortunei* gills, since the morphophysiological structure of the gills favors microorganism bioaccumulation such as amoebas and viruses [19]. The gills are located into the mantle cavity. The water enters through an aperture formed by the apposition of the mantle, called inhalant siphon [20]. Each gill comprises two demibranchs, a double-lamellar macrostructure, which are built up with several filaments forming interfilament channels. There are ciliary bands on the lateral surface of each individual filament that are known as water-pumping cilia or lateral cilia (lc), responsible for the main water flow and pumping through the interfilament channels [21]. On the abfrontal surface of each filament there are frontal cilia (fc) and latero-frontal cilia or cirri (lfc) located at the frontal margin of each filament frontal surface, in between the lc and fc. The action of the lfc is ascribed to the particles capture, which combined to the fc action, transfer particles from the main water current towards the marginal food groove and then to the labial palps [21, 22]. Thereby, in addition to the breathing function, this organ fulfills the capturing and transportation of particles [20].

The comprehension of the invasive mussels' morphology can reveal their role in the ecosystems, and also assist us to better decide on the methods to control their population in invaded sites [23]. A throughout morphological description of the *L. fortunei* anatomy has been reported [20]. Additionally, Paolucci and co-workers [24] reported the association between genetic variability and macro- and micro-structural morphology of *L. fortunei* populations across South America. However, little information about the ultrastructure at the cellular level and the physiology of the gills of the golden mussel is available thus far. In this current work, for the first time, we promoted the ultrastructure characterization of the gills epithelium of *L. fortunei* to better understand the morphophysiology aspects of the organelles in these structures which are one of the most important for their survival as responsible for breathing and feeding.

Results

Gills microstructure

The *L. fortunei* gills have a large surface area fitting the mantle cavity space. Each pair of gills has a leaf-like shape (Figure 1) and is located at both sides of the viscera. Each gill comprises two demibranchs, known as inner- and outer- demibranch, in a double lamellar structure joined by the ctenidial axis (Figure 2.a).

At the margins of abfrontal surface of each filament we could observe the ciliary projections called laterofrontal cilia (lfc) (Figures 3 and 4). Ciliary discs (cd) were observed at the lateral surface of the filaments (Figure 3); they measure about 16 x 10 µm and cross-connect individual filaments. The additional TEM images file shows that the lfc have a microtubule cytoskeleton, the axoneme with the type 9+2 (see Additional file 2). This cytoskeleton structure also applies for the lateral cilia and ciliary discs. Frontal cilia (fc) are located on the abfrontal surface of the filaments (Figures 4.b and 5.a,c) and measured 880 ± 150 nm long, with a diameter of 85 ± 15 nm. The frontal cilia seem to protrude from the epithelium and not to have the same cytoskeleton structure as the lc and lfc. We could not observe pro-latero-frontal cirri (p-lfc) in between the lfc and fc in the *L. fortunei* specimens. Several particles with few micrometers in size were observed attached to the lfc (Figure 3.c,d).

Gills ultrastructure

TEM images show the epithelial cells in sections longitudinally to the lateral surface of demibranch filament (Figure 5). In Figure 5.a many lfc lay longitudinal to the demibranch filament suggesting the lfc are stiffer, as also observed in Figure 4.a. In Figure 5.c the lfc are observed in cross-section, which means mostly are bent, corresponding the their position as shown in Figure 4.c,d. It was possible to observe many mitochondria in the region close to the insertion site of gill cilia (Figure 5.b,c). The mitochondria in Figure 5.c are more easily seen, with much sharper lamellar cristae when the lfc are bent, than in Figure 5.b when the lfc are stiffer.

Another organelle found in the apical region was the smooth endoplasmic reticulum (Figure 5.d). Surrounding the smooth reticulum, we could observe several nearly 70 nm electron dense corpuscles, the glycogen granules. In addition, it is possible to observe the higher number of vesicles (201 ± 29 nm) possibly containing mucus in the Figure 5.b,c.

Discussion

The detailed description of the mucociliary epithelium of *L. fortunei* gills adds new layers of complexity to the biological invasion process caused by the presence of this bivalve in the environment. The distinction of positioning, microstructure and inference of functioning of the multiple types of cilia (fc, lc and lfc) present in this epithelium sheds light on the morphophysiology of gills, which interacts with various types of particles, including chemical compounds used for antifouling treatment to combat *L. fortunei* macrofouling. The ultrastructure characterization of this epithelium becomes crucial to manage the invasion process.

Gills size of the mussels used in the present work did not differ significantly from the population of *L. fortunei* found in waters with high total suspended solids (57.9 ± 23.4 mg L^{-1}) reported in the literature [24]. Our data showed a slightly longer mean cilia length of laterofrontal cilia for the specimen VG, and a slightly lower mean filament width for the specimen PR compared with the data reported by Paolucci and co-workers [24]. The morphometric differences between *L. fortunei* populations, especially gills area relative to shell morphology that is negatively correlated to total suspended solids, has been assigned to their phenotypic plasticity rather than genetic variabilities [24]. We have not assessed the gill and shell areas, but morphometric differences found for VG and PR specimens might be due to different environmental conditions they had to grow and adapt.

We could also find organelles possibly transporting mucus in the epithelial cells. As described for the Brazilian endemic bivalve *Diplodon expansus* (Küster 1856) [25], the production of mucus in this region of the gills might be associated with lubrication, in order to reduce the friction between the water and the epithelium, providing protection. In *D. expansus*, the mucus layer is highly viscous and difficult to hydrate and, consequently, difficult to remove from the epithelium, ensuring the efficiency of this lubricant. It is also possible that the mucus in the bivalve gills cilia change the local fluid mechanical properties, but it is not yet clear whether the particles can be kept in this confined local current produced by the cilia beating without the intervention of mucus [26]. Moreover, this mucus plays an important role in the discrimination of captured particles to be rejected or ingested [13]. The particles capture and transport mechanisms in filter-feeding mollusks are almost exclusively ciliary dependent. The lateral cilia are responsible for pumping water through the gill interfilament channels towards the suprabranchial cavity [21, 26]. One of the functions of the lfc is ascribed to the particles capture [13, 21]. The lfc are also responsible for particles transfer from the main water current to the anterior side of the gill filament. That transportation is accomplished by the lfc [22] or through currents produced as the lfc beat against the main water current [21]. Captured particles are then transported towards the marginal food groove by the metachronal waves [27, 28] generated by the action of frontal cilia. Particles' capture and transportation seem to be autonomous mechanical processes. On the other hand, particles rejection or ingestion is based on physicochemical interactions that can be sensed in the labial palps [13, 26].

The particles selection mechanism is not fully resolved. Some studies show that it depends on particles' characteristics such as size, shape, and surface properties, which affect their ingestion or rejection [29, 30]. The rejected particles are bound to cohesive mucus, deposited in specific places in the mantle and then transported to the cilia, for their expulsion as pseudofeces [31]. Additionally, the mucus covering feeding organs can mediate particles selection [13]. However, it is not yet clear whether the mucus of the bivalve gill filaments play a role in the particles capture mechanism itself [21].

Our data suggest that the mucus in *L. fortunei* gill filaments may be correlated to the lfc stroke. A larger number of mucus-containing vesicles in the epithelium cells were observed when the lfc were bent (Figures 5.c,d). Conversely, less mucus-containing vesicles were observed when lfc recovered their stroke, being longitudinal to the gill filament (Figure 5.a,b). The TEM images suggest the mucus are packed and sent to the apical region, and indeed we observed mucus-like vesicles above the epithelial cells in

between the lfc (Figure 5.c). This may be an ongoing process in which the mucus carried by vesicles towards the gill cilia might be available to interact with the surface of an upcoming particle that will be captured and further transported to the labial palps to be physicochemically sensed and discriminated [13, 26], and then rejected or selected by the feeding organs [13]. The mucus produced by epithelial cells are modified by Golgi complex, near the nucleus, which plays a key role in the sorting of newly synthesized and recycled molecules towards their final destinations [32], the apical region of the epithelium, as can be seen in Figure 5.b,c. The TEM images also showed mitochondria with extensive lamellar cristae arranged in parallel juxtaposed sheets that occupy most of the organelle volume (Figure 5), which is common of highly energy-demanding tissues [33] such as the gill ciliary epithelium [34]. The smooth reticulum was also present, which among several cellular functions is the participation in glycogen metabolism [35], and the glycogen granules are important components for the bivalve metabolism [36]. All this energetic apparatus is related to the morpho-functional structure of the ciliary epithelium and allows us to infer the correlation between the mucus in gill filaments to the stroke of laterofrontal cilia. Such correlation is quite hard to confirm by single TEM sections, though. This would require a 3D reconstruction of the ciliary epithelium at high spatial resolution. This will be further investigated by volume electron microscopy techniques to better understand such dynamical process as this.

Conclusions

The understanding of the *L. fortunei* morphology is the first step towards the establishment of strategies to control this invasive species, and to the authors knowledge this is the first time high resolution ultrastructure of *L. fortunei* gills epithelium have been reported. Our data showed the microstructure of the gills filaments and cilia in high spatial resolution and also evidence of the production and release of mucus-like vesicles in ciliary cells, which may highlight mechanisms of selection and discrimination of particles to be ingested or rejected by mussels. Our findings may contribute to the understanding on how mucus in gill filaments act in the process of the particle selection, which might have implications for further studies to establish new strategies of population control of this invasive species.

Methods

Gills preparation

Specimens of *L. fortunei* were collected in February 2019 in the fish farming reservoir of the Volta Grande, border between the states of Minas Gerais and São Paulo, Brazil (VG; 20°01'54.0"S, 48°13'10.0"W) where measured water parameters were 29.2 ºC, pH 7.5, dissolved oxygen (DO) 3.2 mg L^{-1}, and turbidity (NTU) 0.1. They were packed in cloth bags (to decrease overlapping individuals) and during transportation they were submerged in water at constant aeration. In our laboratory at the *Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas* (CBEIH), the animals were acclimated for 3 weeks in aquarium with 36 L capacity containing artesian water well, pH 7.7, dissolved oxygen 6.8 mg L^{-1}, and turbidity (NTU) 1.68, at constant aeration and temperature 18 ºC to minimize stress. After acclimation, the mollusks were

kept under the same conditions and temperature of 22 ± 1 ºC. For the purpose of this work, one approximately 1.5 cm long bivalve was taken out of the aquarium so their valves could be kept partially open. Next, the specimen was submerged in a 1.5 mL Eppendorf® tube filled with modified Karnovsky fixative solution (paraformaldehyde 2.0% and glutaraldehyde 2.5%). The VG specimen was kept into the fixative solution for 3 days. The gills were collected afterwards to further process them for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The collected gills were then placed into 1.5 mL tubes filled with 0.1 M phosphate buffer solution (PBS). In this present work, we also used unreported TEM and SEM data of the gills from another individual that was collected in a different place, in the Paranaíba river (PR), downstream the confluence with Barreiro river, near the municipality of Paranaíba, Mato Grosso do Sul, Brazil. The sample preparation details about this latter specimen can be found in Andrade et al. (2015).

Scanning electron microscopy (SEM)

Right before the secondary fixation, the excess volume of PBS in the tube was first removed and replaced by an appropriate volume of a fresh 0.1 M PBS and incubated for 10 min. This washing process was performed three times. The PBS was then replaced by an appropriate volume of 1% osmium tetroxide (OsO_4) in 0.1 M PBS (pH 7.3 ± 0.1) in the fume hood and incubated for 1 h in darkness and room temperature (RT). The sample was with 0.1 M PBS and incubated for 10 min, for three times. The excess PBS was removed and replaced by 1% tannic acid ($C_{76}H_{52}O_{46}$) solution and incubated for 20 min at RT. After washing with PBS for three times, the solution was replaced by 1% OsO_4 solution and incubated for 1 h in darkness and RT. The samples were then washed in distilled water three times and dehydrated in a sequence of alcohol solutions (35%, 50%, 70%, 85%, 95% and 100%), 10 min each. The last step with absolute alcohol was performed twice. The sample was critical point dried with CO_2 (using a Leica EM CPD 030), placed on an aluminum SEM stub with a carbon tape and finally coated with gold nanoparticles (5 nm thickness) in a sputter coater (Bal-Tec MED 020). The VG sample was analyzed in a field emission scanning electron microscope FEI Quanta 200, operated at 5 kV and 15 kV. The SEM images of the PR sample were acquired using the same microscope but at 20 kV and low vacuum mode (0.30 Torr).

Transmission electron microscopy (TEM)

For TEM analysis, sample was firstly washed for three times in 0.1 M PBS, 10 min each. PBS was replaced by 2% OsO_4 in 0.1 M PBS (pH 7.3 ± 0.1) in the fume-hood and incubated for 2 h in darkness and at RT. The sample was then washed with deionized water four times, 10 min each. Distilled water was then replaced by 2% uranyl acetate ($C_4H_8O_6U$) solution in darkness and incubated overnight in darkness at 6 ºC. The samples were again washed with deionized water, three times, 5 min each. Samples were dehydrated as described erlier. Absolute alcohol was replaced by acetone and incubated 20 min. Acetone was then replaced by Epon™ resin in three steps, using different mixtures of acetone/resin (2:1, 1:1, and 1:2). In each of these three steps, the tube was enclosed and homogeneously agitated (using a Norte Científica NH2200) for at least 2 h. After drying the excess acetone/resin with a filter paper, the samples

were transferred to a new polypropylene tube containing Epon^{TM} resin prepared with DMP-3 (Sigma Aldrich) and incubated at 40 ºC for 1 h. The gills sample was carefully embedded so to section it longitudinally to the lateral surface of gills filament. The ultrathin (60 nm thick) sections were obtained using an ultramicrotome (Leica EM UC6) and a diamond knife Ultra 45º (Diatome), with the sections being transferred to C-film Cu-TEM grids. Sections were post-stained using a 2% uranyl acetate and lead citrate. TEM analysis was performed in a thermionic W-filament transmission electron microscope FEI Tecnai Spirit G2-12 BioTwin, operated at 120 kV. SEM and TEM sample preparations and their analysis were performed at the Center of Microscopy at the Universidade Federal de Minas Gerais (UFMG).

Structural analysis

The micro-morphological analysis of the *L. fortunei* gills structure was performed using SEM images. TEM was used to describe the ultrastructural morphology of the ciliary gill epithelium. When suitable, measurements of the gill filaments were performed using transmitted light microscopy images (Additional file 2), taken in a Leica DM4500 microscope, with the same plastic embedded blocks for TEM preparation. Filament width, interfilament space, and the filament linear density (number of filaments per linear distance) were obtained from SEM images. Cilia length was obtained in SEM images, and the cilia diameter and cilia linear density (number of cilia onto the filament over the linear distance along the filament) using the TEM images. Measurements were performed using the free softwares FIJI / ImageJ v. 1.52p (Wayne Rasband - National Institutes of Health, USA) and the DigitalMicrograph® v. 3.41.2916.1 (Gatan, Inc).

Declarations

Ethics approval and consent to participate: The experiments have been conducted in compliance with the Brazilian law nº 11.794, of 08/10/2008, (available at http://www.planalto.gov.br/ccivil_03/_ato2007-2010/2008/lei/l11794.htm), that concerns laboratory and field experiments involving animals and following the orientation of the Ethical Committee of the Federal Universidade of Minas Gerais (UFMG), which follows the Brazilian regulations on the matter (available at https://www.ufmg.br/bioetica/ceua/).

The law ruling of the animal experiment is concerned about those animals pertaining to the Phylum *Chordata* and the subphylum *Vertebrata*. For all the other cases, the submission of the proposal to the above-mentioned ethical committee is waived.

Even though there are no specific rules about experiments using invertebrates our studies were also carried out in compliance with international standards in ethical research and recent scientific concerns, as those expressed in Drinkwater et al (*Methods Ecol Evol*, 2019, 10(8):1265-1273. doi.org/10.1111/2041-210X.13208), and ASAB (*Animal Behaviour*, 2020, 159:I-XI. doi.org/10.1016/j.anbehav.2019.11.002).

Our experiments were performed using individuals of the species *Limnoperna fortunei* (mollusks of the class *Bivalvia*). The collection, transport and maintenance of the mollusks in the laboratory have been authorized by the environmental agency ICMBio, linked to the Ministry of the Environment (and can be

accessed in https://drive.google.com/file/d/1SVsAlcTtghxozTmYpyVu9JhEvKX0eMQx/view?usp=sharing).

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Competing interests: We declare no competing interests.

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Author's contribution: Mr. ETFF performed the electron microscopy analyses and compiled the data. Ms. AMSM collected the samples at the CBEIH laboratory and did the pre-fixation. Mr. ETFF, Ms. AMSM, and Mr. RSdP contributed to the data analysis and to the all written part of the manuscript. Ms. GRA helped in the manuscript review and agreed to give non-published electron microscopy images of *L. fortunei* gills she investigated earlier. Mrs. MDdC is the project manager and Professor Mr. PSA the coordinator of the R&D Aneel/Cemig GT-604. Professor Mr. AVC is the research group leader who had the idea of studying the *L. fortunei* gills and revised this manuscript. Professor Mrs. ECJ did the throughout review of the manuscript. All the authors read and approved the manuscript.

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Table

Table 1. Measurements of the gills structure variables (mean ± standard deviation).

Specimen	Filament width (µm)	Interfilament space (µm)	Filament linear density (N / µm)	Cilia length (µm)	Cilia diameter (nm)	Cilia linear density (N / µm)
VG	*17.4 ± 1.9	*17.3 ± 2.4	*19.5 ± 1.7	15.1 ± 1.1	246 ± 11	18.4
PR	10.4 ± 1.5	20.5± 0.7	40.5 ± 0.8	10.7 ± 1.1	247 ± 10	15.7

* Measurements performed with transmission light microscopy images

Figures

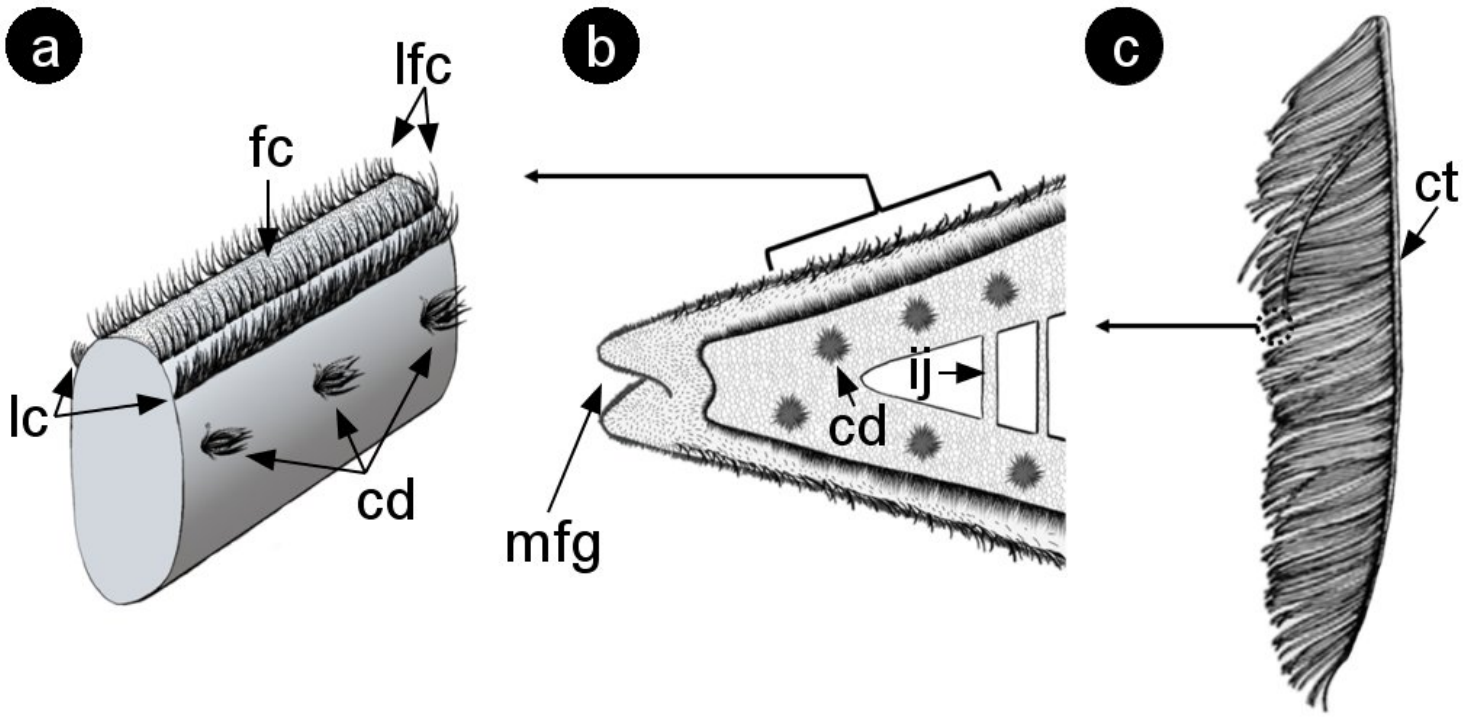

Figure 1

Representation the L. fortunei's ciliary gill showing the frontal cilia [fc], lateral cilia [lc], laterofrontal cilia [lfc], ciliary discs [cd], marginal food groove [mfg], interfilament junction [ij], amd ctenidial axis [ct]. (a) View in perspective of a portion of one filament showing its abfrontal surface at the top ridge and its lateral aspect. (b) Longitudinal view of the filament showing its lateral aspect. (c) Dorsal view of the gill.

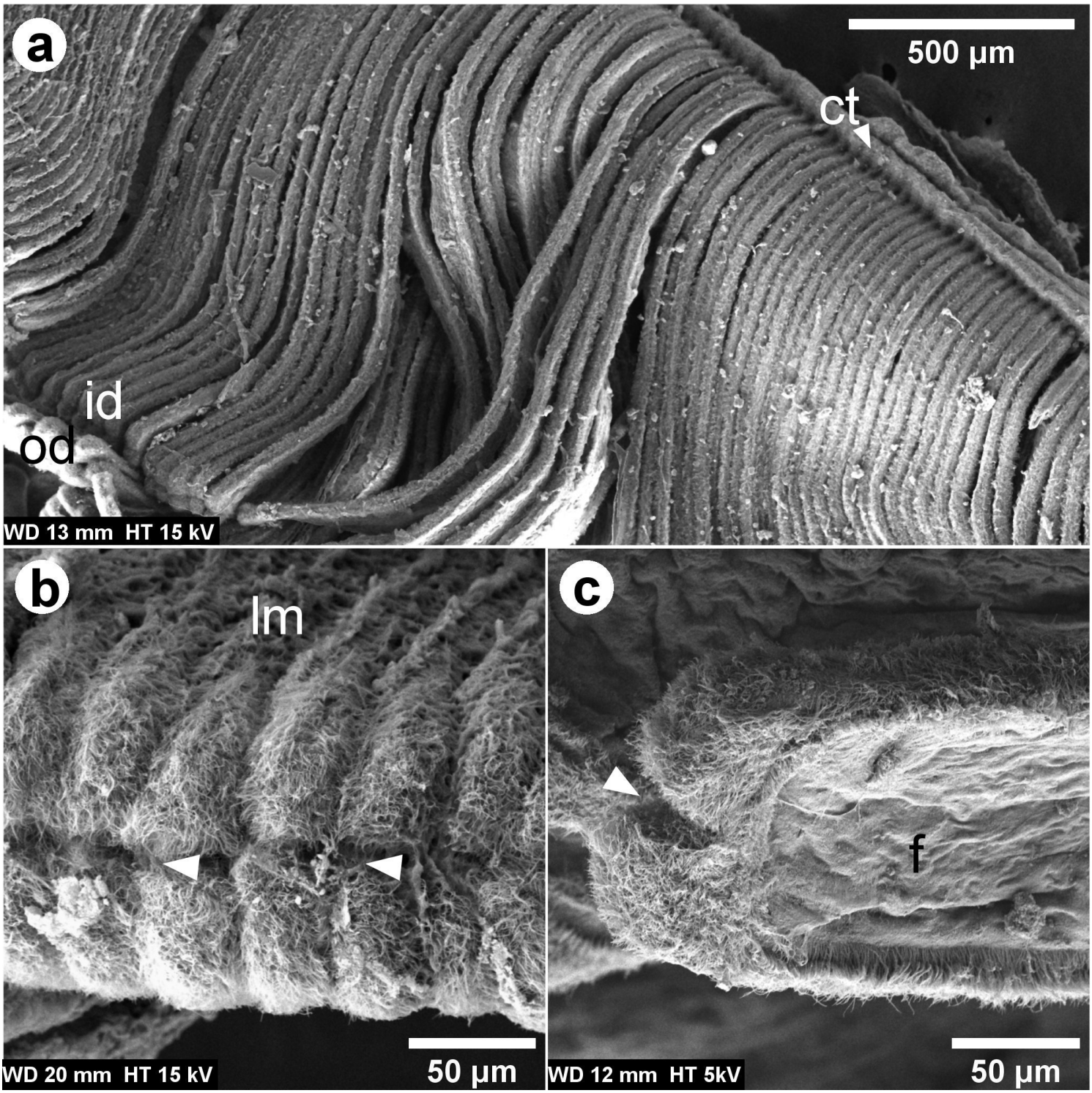

Figure 2

Secondary electron SEM images of the L. fortunei gills. (a) Dorsal view of the gill showing the lamellae, the inner- and outer- demibranch [id and od] joined by the ctenidial [ct] axis. (b) Frontal view of one lamella [lm] showing its anterior aspect. (c) Longitudinal view of one filament [f] showing its lateral aspect with a W-shape. The marginal food groove at the lamela's edge is pointed out by the arrowheads in images (b,c).

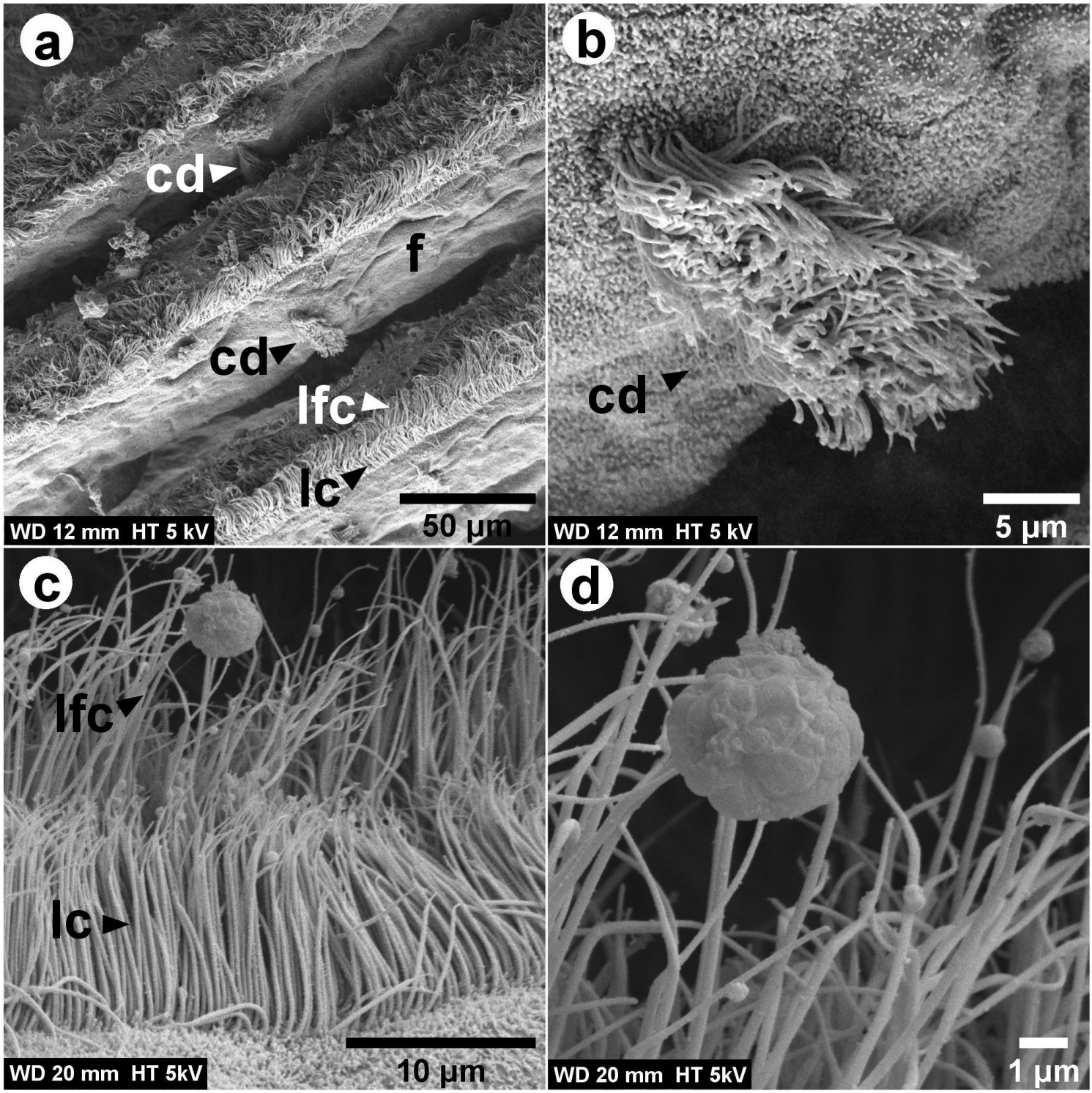

Figure 3

Secondary electron SEM images of the L. fortunei gills. (a) View in perspective of a set of filaments [f], showing ciliary discs [cd], lateral cilia [ld] and laterofrontal cilia [lfc]. Images (b) and (c, d) show in detail a cd, the lc and lfc, respectively. Several particles with few micrometers in size were observed attached to the lfc.

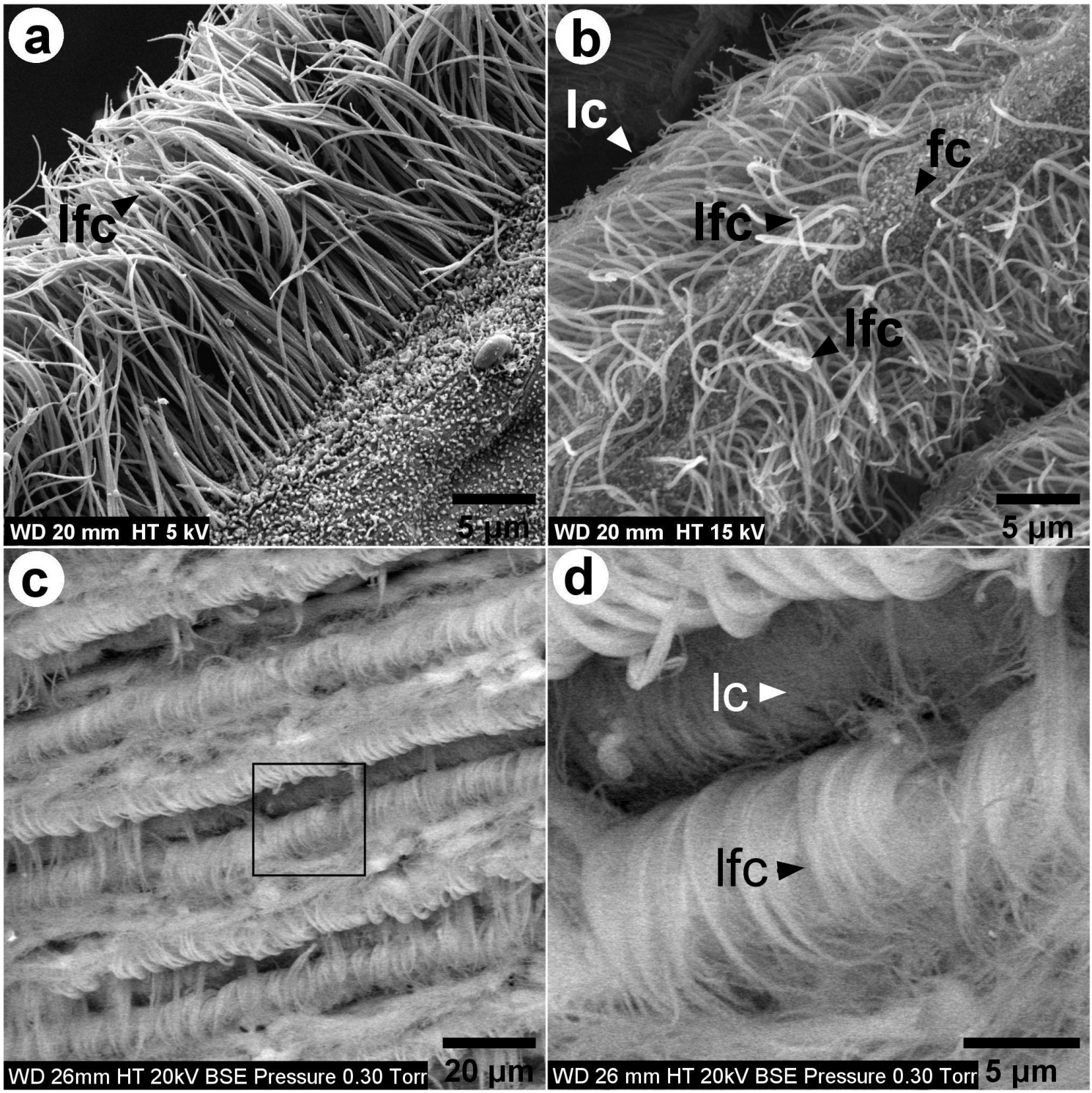

Figure 4

(a, b) Secondary electron SEM images and (c, d) backscattered electron images of the L. fortunei gills. (a) Longitudinal view of one filament at higher magnification showing its lateral aspect and the detailed laterofrontal cilia [lfc] at the top frontal margin. (b) Abfrontal surface of one filament showing the lateral cilia [lc] and the frontal cilia [fc] on apical region of epithelium in between the lfc. (c) Abfrontal surface of the filaments. (d) Higher magnification image of the region marked with the square marked in (c), showing the lfc and lc.

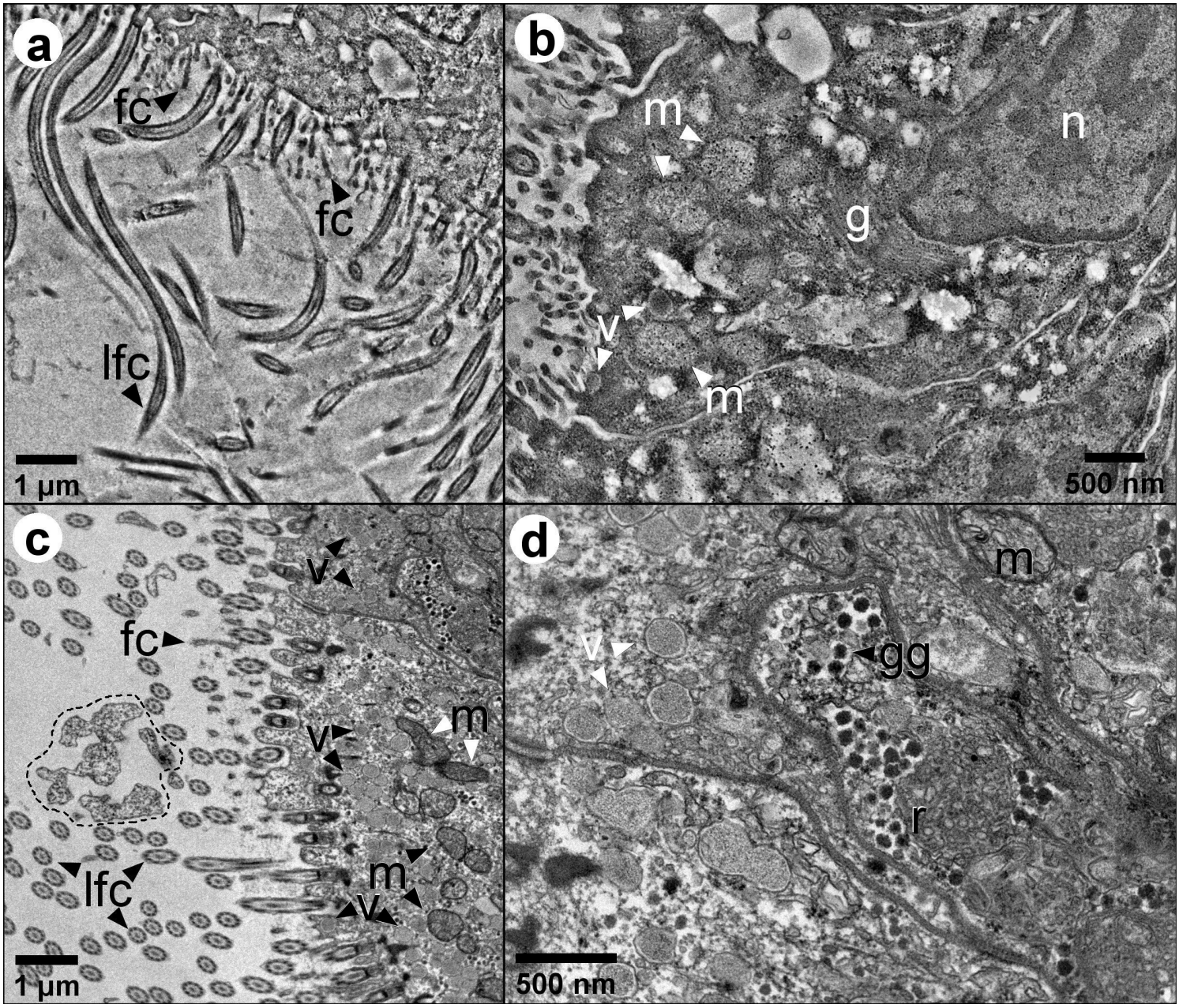

Figure 5

Bright field TEM images of sections longitudinal to the lateral surface of L. fortunei's gill filament. In image (a), the many laterofrontal cilia [lfc] and frontal cilia [fc] are observed longitudinal to the gill filament, and in image (c) the lfc are observed in cross-section. Images (b) and (d) show the epithelial cells at higher magnification, displaying the nuclei [n], mitochondria [m], Golgi complex [g], mucus containing vesicles [v], smooth reticulum [r], and glycogen granules [gg]. The region highlighted in image (c) shows possibly mucus in between the lfc. The arrowheads in images (b, c, and d) point some vesicles, mitochondria, and glycogen granules.

Supplementary Files

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