

## ORIGINAL PAPER

# The Extraordinarily Rare Ciliate *Legendrea loyezae* Fauré-Fremiet, 1908 (Haptoria, Ciliophora)



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Diverse and dynamic communities of ciliates and other microbes thrive in the natural environment, driving the functioning of aquatic ecosystems. Many microbes are present in very low numbers or are dormant in the 'seedbank', escaping detection in environmental surveys and, consequently, remaining underexplored. Here, we report an extraordinarily rare ciliate that was discovered after persistent exploration of freshwater anoxic sediments - *Legendrea loyezae* Fauré-Fremiet, 1908, a member of the Family Spathidiidae, Order Haptorida. In this study, we present the sixth account of the ciliate since 1908 and reveal its phylogenetic position with the first 18S rRNA data for the genus. We explain the key morphological features of the species, describing a remarkable behaviour in which the ciliate "shapeshifts" due to its ability of controlled full extension and retraction of its tube-like tentacles. Our results shed light on the similarity of *L. loyezae* to another ciliate that was first described as *Legendrea bellerophon*, later moved under a new genus and named *Thysanomorpha bellerophon*. We question the validity of this taxonomic decision and, based on morphological characters and tentacle movement, we propose moving *T. bellerophon* back under *Legendrea*. This study demonstrates how continued and persistent exploration of natural habitats lead to the discovery of microbial communities and species.

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**Key words:** anaerobic ciliates; *Lacerus*; microbial diversity; *Thysanomorpha*.

## Introduction

Microbial communities in natural habitats are dynamic and diverse and a significant proportion of microbial species can be found in the sediment

of lakes and ponds. This is particularly applicable to microbial eukaryotes such as ciliates, which are well-defined at morphological level and show great diversity. However, as only a limited number of ciliate niches are available at any one time, the very

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rare ciliates may not be engaged in population growth, awaiting the arrival of suitable environmental conditions for growth and reproduction (Finlay and Esteban 1998), or they may be present in such small numbers that they remain undetected in environmental surveys. As in the case of this study, sampling a precise location methodically for a period of time may lead to new observations as the habitat changes and creates new niches.

Microbes run ecosystem functions, their extraordinary abundance secures their crucial position within food webs as regenerators of nutrients (Finlay and Esteban 1998), and they hold potential for discovery of new biotechnological compounds (Paoli et al. 2022) as well as new taxa. By interacting with environmental physical and chemical factors, microorganisms promote a constant turnover of microbial niches that are filled by the rare and dormant species from the 'seedbank; for example, when aerobes proliferate and use up all the available dissolved oxygen in the water, it results in the death of aerobes but opens a new niche for anaerobes (Esteban and Fenchel 2020; Finlay and Esteban 1998). Here, we report an extraordinarily rare ciliate that was discovered after persistent exploration of freshwater anoxic sediments - *Legendrea loyezae* Fauré-Fremiet, 1908.

*Legendrea loyezae* is a freshwater anaerobic ciliate that has been reported only five times prior to this publication since its first description in 1908 (Fauré-Fremiet 1908). The ciliate is distinguishable at the first glance due to its remarkable morphological feature of bearing numerous finger-like extensions (tentacles) of different lengths, located towards the rear of the swimming cell. Previous descriptions of this ciliate differ from each other (Fauré-Fremiet 1908; Kahl 1930; Kreutz 2014, 2021; Penard 1914), and the cause of the morphological differences was never truly understood before. The position of the tentacles was represented emerging from different parts of the cell, but the ability to extend the tentacles in full was never seen nor described. Here we present morphological details of two populations of *L. loyezae* retrieved from two freshwater ponds separated about 25 km from each other. We also describe the full extension and retraction of the finger-like appendages (tentacles) for the first time and present the first partial 18 S rRNA gene sequence for this species. We provide morphological justification for changing the genus *Thysanomorpha* back to *Legen-*

*drea*, and we highlight the importance of taxonomic surveys and the continued exploration of the microbial world.

## Results

**Diagnosis of the genus *Legendrea*:** cell with retractable and extendable tentacles, located either around the cell in variable number, or situated more posteriorly. In some species, the contracted tentacles appear like protruding papillae with extrusomes at their tip. In other species, the retracted tentacles appear like finger-like projections, with extrusomes at their tip. The ciliates have two different forms: stationary and free-swimming, respectively, each with different morphology. The free-swimming form is oval and characterised by the contraction of the tentacles whilst the ciliate moves in the environment. The stationary form is the feeding phase; in it, the ciliates remain motionless amongst sediment particles, the cell shape is flattened, and the tentacles are slowly extended until fully stretched. The ciliates remain in that position waiting for the prey. One or more contractile vacuoles present. Uniform ciliature distributed over the surface of the cell, apical cytostome, and brosse present. Intracellular prokaryotes may be present. Macronucleus variable in shape. Micronucleus present but not always observable. Free-living anaerobic ciliates that thrive in freshwater anoxic sediments.

**Type species of the genus:** *Legendrea loyezae* Fauré-Fremiet, 1908.

**Diagnosis of *Legendrea loyezae*.** Cell size 75–120 µm in length and 40–55 µm in width. Cell is ovoid, slightly flattened; uniform somatic ciliature and complete oral bulge with extrusomes arranged in short, dense, oblique rows; one single vacuole located at the posterior end of the cell; one elongated macronucleus, usually horseshoe shaped; characteristic finger-like tentacles located towards the cell posterior, but location varies in relation to their contraction status; number of tentacles 15 to over 30; the tentacles can be extended when the ciliate is stationary and contracted when the ciliate reverts to swimming. Free-living anaerobic ciliate that thrives in oxygen-free freshwater sediments. Presence of intracellular prokaryotes in the ciliate cytoplasm.

**Gene sequence:** One partial 18S rRNA gene sequence from *Legendrea loyezae* was deposited in GenBank and has accession number: OP352778.

Description of *Legendrea loyezae* (Figs 1-3; [Supplementary Material](#))

We were able to observe four cells of *Legendrea loyezae*, found within the timeline of late 2021 and mid 2022. We found the first cell in a two-month-old sample, and we were able to fix it for genomic analysis. However, our most exciting discovery came when we found our second cell in a sealed slide. These slides are prepared by using some regular sized microscope slides and large coverslips with petroleum jelly streaks on the edges. Simply, the coverslips were pushed on a drop of sample so that the petroleum jelly sealed the slide. The slides were prepared for the longer observation of metopids in the anaerobic sediment, however a quick first scan of one of the slides revealed a *Legendrea loyezae*. The cell was swimming with trailing tentacles and appeared healthy. Upon initial observations and video capturing, the slide was put in a humidity chamber and the same *L. loyezae* cell was observed through the course of five days. During the first day, the swimming cell lodged itself into some debris, mouth pressed against the coverslip and became motionless; however, methodical swirling of the cytoplasm was apparent, meaning that the cell was alive even though staying stationary. At this point we were not able to get a clear view of the tentacles because of their position under the debris and the cell body, so we left the slide in the chamber hoping to see the cell alive the next day.

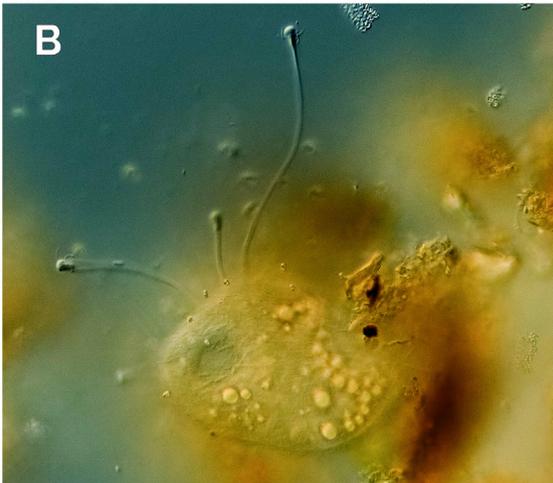
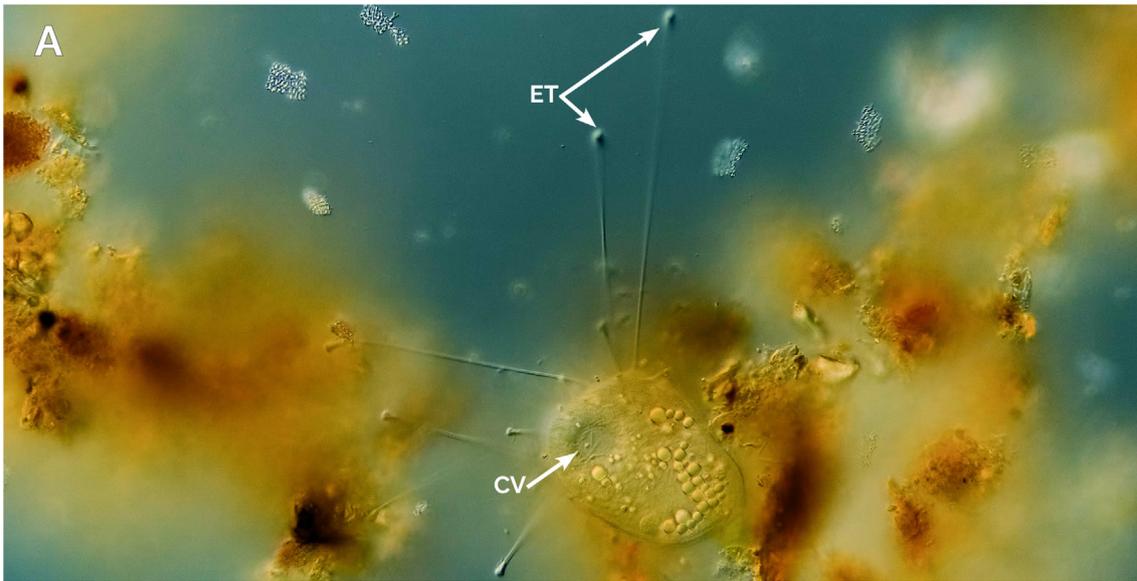
When we investigated the cell the next day, we found out that the cell was still at the same place, but its position had slightly changed; it was lying flat on its side, and we could see the way the tentacles were behaving. What we were seeing was never observed by any of the aforementioned authors, i.e., [Fauré-Fremiet \(1908\)](#), [Penard \(1914\)](#), [Kahl \(1930\)](#) or [Kreutz \(2014, 2021\)](#) who found *L. loyezae* before. The tentacles of our cell were fully extended ([Fig. 1A](#)). Upon extension, the tentacles became thinner and the cilia around the tip of the tentacles were active and beating, allowing the tip that carries the extrusomes move like a “fish bait.”

We observed the cell in this position with extended tentacles for two more days. Overall, the position of the tentacles changed and some of them extended even further but the ciliate remained motionless. When fully extended, the tentacles are very narrow and can be, at least, twice the length of the ciliate ([Fig. 1A](#); [Supplementary Material](#)). Although we haven't seen a proof of it, we presume

the extended tentacles is how *L. loyezae* captures its food - the ciliate waits for an appropriate prey to come in contact with the tentacles, similar to the ciliate *Actinobolina*'s behaviour, i.e., once the prey is captured by the discharged extrusomes it is moved by the joint action of cilia and tentacles to the cytostome, where it is ingested (personal observations of *Actinobolina*; [Calkins 1901](#); [Holt and Corliss 1978](#)). We tried to get the ciliate moving by slightly tapping on the slide, or in the case of many other ciliates that like to live in light-free environments, by exposing it to strong light. However, *L. loyezae* did not react to any of these attempts, even staying motionless under short bursts of UV light.

However, on the fourth day, while we were recording the ciliate in the same position, a green euglenid *Monomorphina* sp., which was attracted to the microscope light, lodged itself in the debris just next to *Legendrea* which quickly triggered a response from the ciliate. *L. loyezae* retracted its tentacles and started swimming with posteriorly positioned tentacles ([Fig. 1B-E](#)). The entirety of contracting the tentacles took less than 30 seconds. Most probably, *L. loyezae*, as an anaerobe, was reacting to the oxygen produced as by-products of *Monomorphina*'s photosynthesis. We were able to video record the complete tentacle contracting process, it can be accessed and watched via the [Supplementary material](#). After a couple of minutes of movement, once again the cell stayed stationary and extended the tentacles. We observed the cell another day at the same position, but we couldn't find the ciliate on our slide the next day.

Our observations of the extension and retraction of the tentacles can also explain the differences between the published descriptions of the cell morphology ([Fauré-Fremiet 1908](#); [Kahl 1930](#); [Kreutz 2014, 2021](#); [Penard 1914](#)). While *L. loyezae* controls the length of the tentacles, it also somewhat controls the position of them on the cell. When the tentacles are fully contracted, the posterior part of the cell is folded to form an indentation which makes the whole organism appear heart-shaped ([Figs 1-3](#)). However, when the tentacles are fully extended, the indentation rather disappears or becomes less prominent, and the cell's heart-shaped morphology also vanishes. Previous observations of *L. loyezae* were all done when the tentacles were contracted and the cell in motion (swimming), which makes it (as we have witnessed in our preparations) very difficult to pinpoint the precise position of the tentacles and, hence, the contrasting descriptions by the dif-



ferent authors. The particular observation by Penard (1914) echoes the difficulty “In principle, they are still lateral, and the first [tentacle] especially, the most anterior, originate on the left and on the right of the broad face of the body. But very quickly this primitive disposition is lost, and the arms are seen distributed almost everywhere and without order behind the animal, forming a tuft which trails during the locomotion.”

We postulate that Penard (1914) was describing a specimen with partially contracted tentacles. His remark about the lateral origin of the tentacles and then the lateral arrangement of them disappearing is what we see when the cells are forming the indentation while contracting. As the cell contracts the posterior part of the cell folds inwards towards the centre of the cell while keeping some of the tentacle bases inside the indentation which then appear as without an order, previous laterally arranged tentacles become posterior with the forming of the indentation (Figs 1, 2).

All four individuals that we found had cytoplasm full of oil droplets (Fig. 2A); these are spherical structures that we often observe in histiophagus ciliates. The cell size ranged from 70 to 95  $\mu\text{m}$  long, and 35 to 55  $\mu\text{m}$  wide. The macronucleus is large and cylindrical, often curved to form a horseshoe-shape (Fig. 1C, 2E). We couldn't observe the micronucleus in the cells since we avoided using stains to keep our valued cells alive. A single posterior contractile vacuole was observed, that widens when the tentacles are extended (Fig. 1A-C). The cytoplasm of all the cells contained numerous endosymbiotic prokaryotes with different morphological appearances (Fig. 2F, G) indicating that *L. loyezae* may have a symbiotic relationship with one or more prokaryotes, some of which may probably be methanogenic archaea that are common in anaerobic ciliates (Esteban and Fenchel 2020; Fenchel and Finlay 2018; Lewis et al. 2020). The number of tentacles (referred to as “toxophores” in Jankowski 2007) ranges from 18 to more than 30, each with 5–8 cilia in a ring around each tentacle's terminal tip (Fig. 2D). We are not sure if the tentacles contain

two types of extrusomes, as reported by Kreutz (2014, 2021). In our view, there is one type of extrusomes only, long, needle-shaped and curved, located at the distal portion of each tentacle, becoming crammed at the tip of the latter and forming a domed papilla (Fig. 2D). As we could not carry out any electron microscopy examinations the nature of the extrusomes remains unknown; they may be toxicysts, as described in *Actinobolina* (Holt and Corliss 1978), which is also a ciliate with retractable tentacles. In *L. loyezae* contracted tentacles appear to have an internal single vacuole from which the long extrusomes protrude toward the tip of the tentacles (Fig. 2D); contracted tentacles also show foldings of the membrane, gathered at their base (Fig. 2D, 3G-4). Fully extended tentacles are thinner and appear empty (probably as result of the internal vacuole) except for the bundle of extrusomes at their distal end (Fig. 1C, 2).

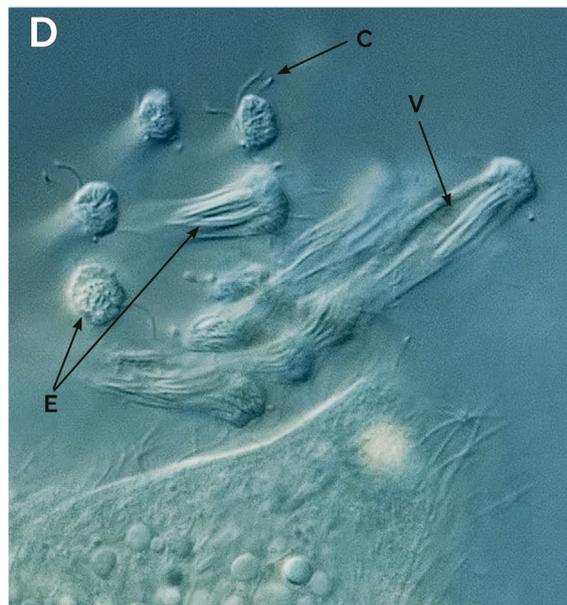
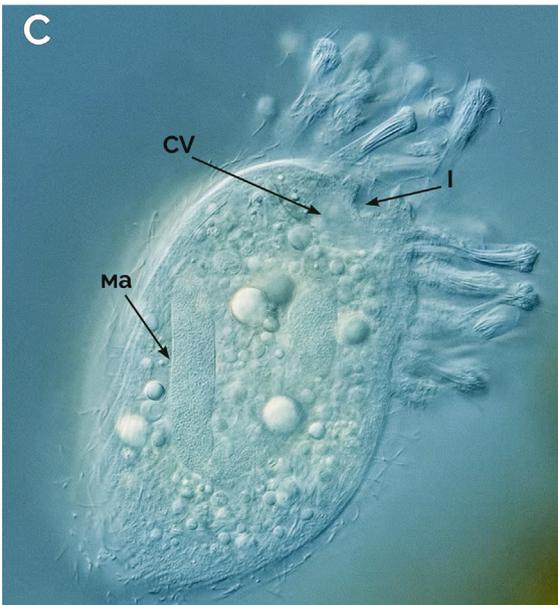
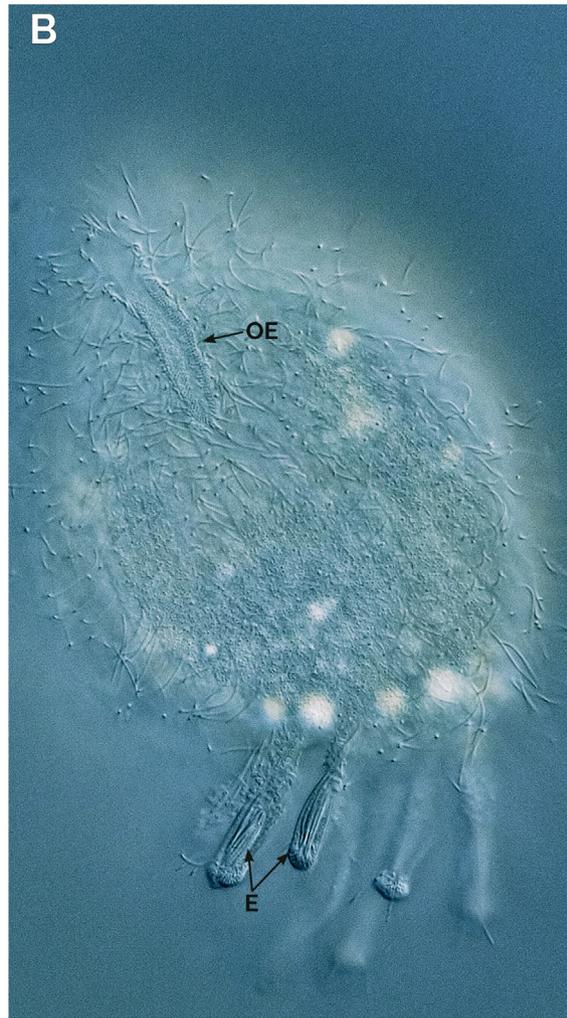
The oral bulge is conspicuous when the cell is viewed ventrally (Fig. 2B), with extrusomes densely arranged in short, oblique rows (Fig. 2B). There seems to be a circumoral kinety, but this feature would need to be confirmed with silver impregnation. Numerous filiform, curved, extrusomes are present throughout the cytoplasm (Fig. 2A). Given the low number of cells that we could handle, silver impregnation was not possible and therefore the number of kineties could not be counted accurately but based on the available video footage (Supplementary Material), the number of kineties is around 20 to 27 (Fig. 2B), but there could be more. This somatic ciliature is uniform over the surface of the cell. There is a brosse formed by three short kineties.

#### Phylogenetic Relationships of *Legendrea loyezae*

Phylogenetic analysis of the partial 18S rRNA gene sequence for *L. loyezae* (Fig. 4) suggests a relationship with members of the Family Spathidiidae, Order Haptorida. The sequence forms a sister group (bootstrap support = 38) to a clade containing three *Epispathidium* sequences. *L. loyezae*'s sequence



**Figure 1.** Differential Interference Contrast (DIC) images of living cells of *Legendrea loyezae*. (A) Cell with the tentacles fully extended. (B) Same cell showing initial contraction of the tentacles. (C) Ciliate with further shrinkage of the tentacles and ready to swim off from the debris spot. (D) and (E) show each side of the same cell, with fully contracted tentacles and in its characteristic swimming motion form. Cell size: 80  $\mu\text{m}$ . CV, expanded contractile vacuole; CT, contracted tentacles; ET, extended tentacles.



also shows as more closely related to *Aperthospathula oktemae* (Family Apertospathulidae) and to a *Arcuospathidium* sp. than other spathidiidae.

## Discussion

E. Fauré-Fremiet (1883–1971)

French protozoologist Fauré-Fremiet published nearly 500 items in his seven decades of scientific life (Nevill 1972). He described many new species and genera including the genus *Legendrea*. Fauré-Fremiet described the genus with the type species *Legendrea loyezae* in 1908 (Fauré-Fremiet 1908).

The publication is in French and Fauré-Fremiet starts his article by noting the rarity of his discovery which "...I was unfortunately only able to see a few specimens..." and mentions the sampling spot as "...in the mud at the edge of the pond Pourras, near Paris...". He makes some remarks on the "infusoria" that usually accompany *L. loyezae* and describes the morphology of his ciliate. French description of the species translates to English (Curds 1982) as; Body shape irregularly oval in outline with broadly round posterior and truncated anterior. Body narrows anteriorly, apical region slants slightly posteriorly from right to left. Unciliated ridge bearing slit-like oral aperture borne upon the slanted apex. Oral aperture supported by a very large basket of trichites which occupies the anterior third of the body. On the left (upper) surface there are about 20 finger-like structures of variable length, each is slightly dilated at its distal tip in which trichocysts are located. These processes are plastic but not capable of controlled movement. Macronucleus elongate. Contractile vacuole either on ventral edge with canals or large and terminal.

*L. loyezae*'s description in Fauré-Fremiet's (1908) article is quite short; no information is given on the etymology of the genus or the species name,

neither mentions the size of the organism, and ends without describing more about the ecology or the behaviour of the ciliate. We do not know if he ever found *L. loyezae* again after his 1908 publication.

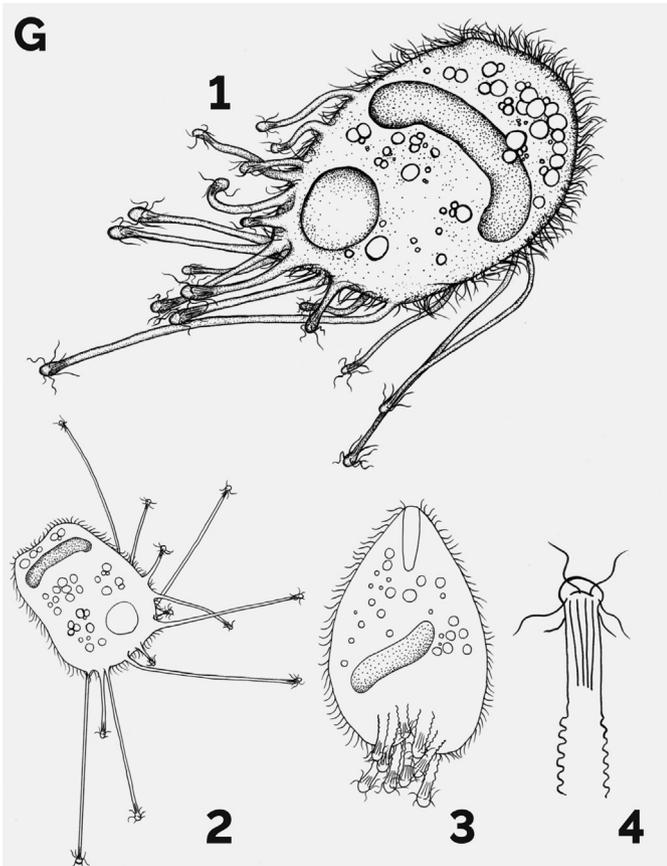
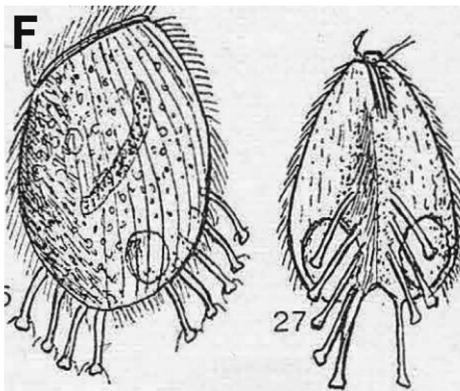
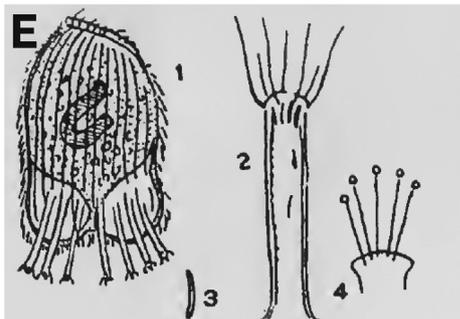
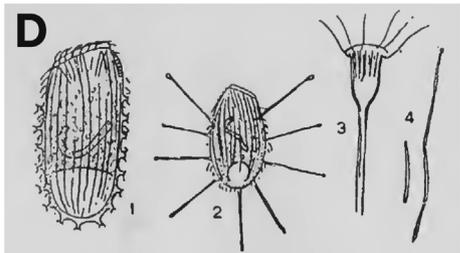
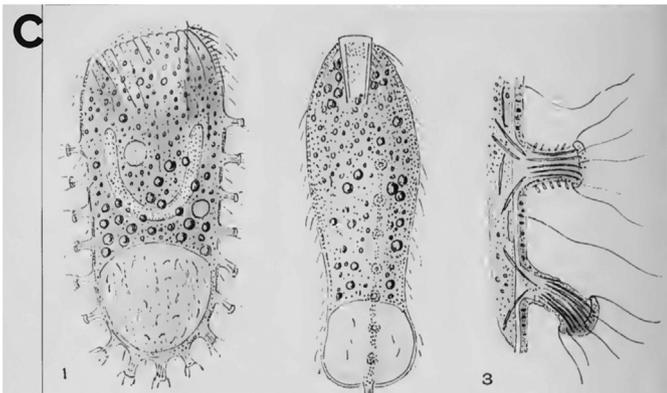
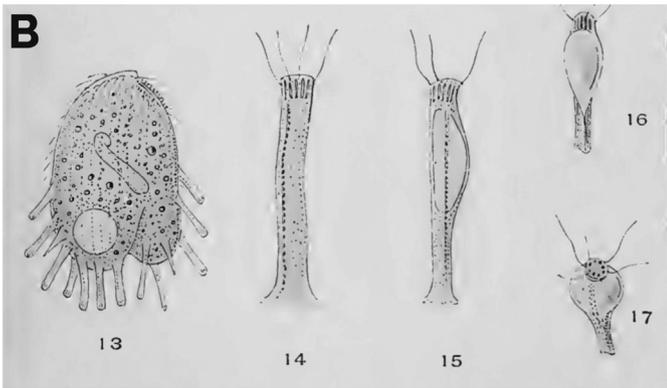
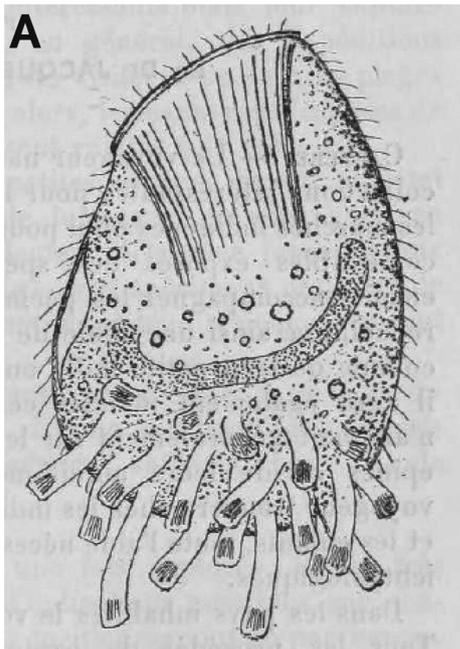
E. Penard (1855–1954)

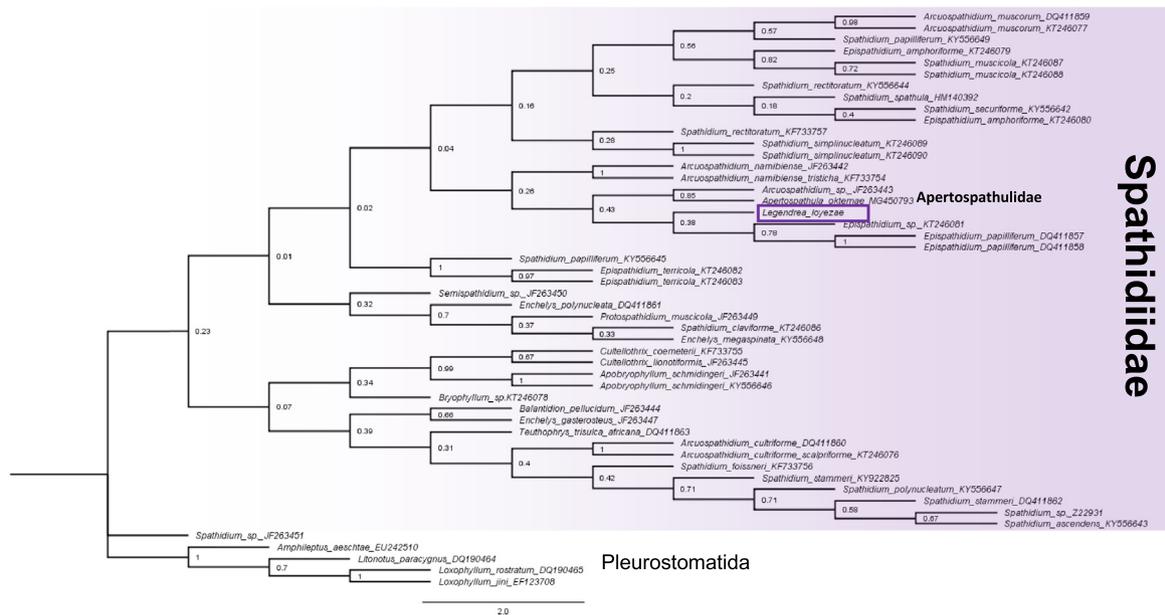
Swiss biologist Eugene Penard's contributions to the field of protistology is unarguably one of the most impactful ones in history (Deflandre 1958). In his long life, Penard described hundreds of microorganisms from colorless flagellates to testate amoebae, rotifers to ciliates. Penard, with his admiration for details and unbelievable skills at microscopy, left hundreds of prepared slides and thousands of detailed drawings. He saw features with his non-immersion objectives (Deflandre 1958) that are even hard to spot with modern immersion lenses with high resolving power. Penard described six new species of the genus *Legendrea*, one in 1914 (Penard 1914), and the rest in 1922 (Penard 1922).

In his 1914 publication, Penard describes a new species he named as *Legendrea bellerophon* (Fig. 3C). This was the second species of the genus *Legendrea* at the time and he also stumbled upon *L. loyezae* in his samples from Geneva. Penard (1914) includes a detailed description of *L. loyezae* in his 27-page-long paper (Fig. 3B), improving Fauré-Fremiet's (1908) description, and comparing the two *Legendrea* species.

As Penard explains, *L. bellerophon* is very closely related to *L. loyezae*, and except for the slightly different size and overall shape, these two ciliates are distinguished easily based on the location, shape and type of the tentacles. Penard (1914) describes the trichocysts location on *L. bellerophon* as lateral "sessile pimples" on the cell, while mentioning that *L. loyezae*'s carries the trichocysts in "arms", i.e., tentacles. Penard explains the position of the "arms" further by "In principle, they are still lateral, and the first especially, the most anterior, originate on the

**Figure 2.** Living cells of *Legendrea loyezae* taken in Differential Interference Contrast (DIC). (A) and (B) different focal planes of the same cell; (A) Cytoplasmic oil droplets, and endosymbiotic prokaryotes of two different (morpho)types; (B) Oral area and somatic kineties. (C) Horseshoe-shaped macronucleus and oil droplets in the cytoplasm. (D) Close up image of the contracted tentacles showing the internal, curved extrusomes (E) and their tight arrangement at the tip of each tentacle. Cell size: 95 µm. C, cilia; CV, collapsed contractile vacuole; D, oil droplets; E, extrusomes in the cytoplasm/tentacles; F, membrane foldings in contracted tentacles; I, indentation formed when the cell retracts the tentacles; Ma, macronucleus; OE, arrangement of the oral extrusomes; P, intracellular Prokaryotes; V, vacuole inside the tentacles.





**Figure 4.** Phylogenetic tree depicting the placement of *Legendrea loyezae*. GenBank accession number: OP352778. The phylogenetic tree has been created using the Maximum Likelihood method and Tamura 3-parameter model G + I (Thompson et al. 1994) using 1000 bootstraps. Bootstraps values are shown in the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 50 nucleotide sequences. There were a total of 1007 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021).

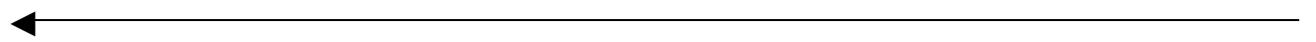
left and on the right of the broad face of the body. But very quickly this initial position is lost, and the arms are seen distributed almost everywhere and without order behind the animal, forming a tuft which trails during the locomotion.”

Penard includes the size of *L. loyezae* which ranged from 70 to 80 µm in length, which matches our observations. He also remarks that *L. loyezae* was much rarer than *L. bellerophon* in the same habitat and that he found 8–10 individuals of *L. loyezae* in the marsh of Rouelbeau, Geneva prior to his 1914 paper. He notes details on the differences between the two species and mentions that *L. loyezae*’s “papillae” (Fig. 3B) are not like “pimples” as in the case of *L. bellerophon* (Fig. 3C, D) but are like “arms” (tentacles). He adds that instead of the tentacles being organized in a lateral way in the case of *L. bellerophon*, they are scattered all over the cell.

Hence, when *L. loyezae* swims the arms trail behind it, Penard notes. This description of the position of the tentacles contradicts, or improves, what Fauré-Fremiet (1908) provided since Fauré-Fremiet mentioned the position of the tentacles on the left side of the cells.

Penard describes that in *Legendrea bellerophon*’s case the “sessile pimples” are capable of controlled extension while *L. loyezae*’s tentacles do not have any controlled mobility and they are not capable of extending out. He adds that they are slightly retractable and gives a detailed comparison of the trichocysts of the two species.

In 1922, Penard publishes further details on *L. loyezae* and mentions that he has been finding the species from time to time since 1914, directing the reader for details on its morphology to check his 1914 publication. However, he did not manage to



**Figure 3.** Line drawings of *Legendrea loyezae* and *Legendrea bellerophon*, respectively. (A) *Legendrea loyezae* after Fauré-Fremiet (1908). (B) *Legendrea loyezae* after Penard (1914). (C) *Legendrea bellerophon* after Penard (1914). (D) *Legendrea bellerophon* after Penard (1922). (E) *Legendrea loyezae* after Penard (1922). (F) *Legendrea loyezae* after Kahl (1930). (G) *Legendrea loyezae* based on our observations; 1,2. Cells with extended and contracted tentacles; 3. Swimming cell with fully contracted tentacles; 4. Tip of a contracted tentacle showing extrusomes, cilia and membrane foldings.

witness the extension and retraction of *L. loyezae*'s tentacles, phenomenon that he did observe and described for *L. bellerophon* (Fig. 3C, D).

#### A. Kahl (1877–1946)

Alfred Kahl was a high school teacher in Hamburg who “accidentally” encountered ciliate studies when his daughter brought home some protozoological literature (Foissner and Wenzel 2004). In less than a decade, Kahl produced an unmatched record of ciliates by combining the existing literature with his nearly 700 new species descriptions.

Kahl published his observations of *Legendrea loyezae* in his first monograph (Kahl 1930). Kahl notes that the size of the organism was 70–90 µm, size that agrees with our observations. He draws attention to the position of the “arms” (i.e., the tentacles) and mentions “arms on the posterior side of the body, gently trailing”. Then he points out that Fauré-Fremiet (1908) and Penard (1914, 1922) showed the tentacles on the lateral surface of the cells, and he adds that in one of his observations “the arms stand on either side of a dorsoventral notch of the body” (Fig. 3F). And he adds that “a new examination is desirable since Fauré-Fremiet and Penard’s description also differ from each other”. Kahl also mentions that he was only able to observe two cells in his “Forgotten Monograph from 1943” (Foissner and Wenzel 2004).

#### M. Kreutz

There are no other reports of *Legendrea loyezae* after Kahl until a very talented microscopist Martin Kreutz finds a single specimen in 2014 (Kreutz 2014), and thereafter several more cells (Kreutz 2021). The cells were found at three different freshwater ponds (Kreutz 2021) in Germany. The specimens found were 70–120 µm in length, bearing 16 tentacles on each side of the cell (i.e., 32 in total); Kreutz (2021) explains that his observations do not seem to agree with the information provided by Fauré-Fremiet (1908), who gave a total of 20, i.e., only 10 on each side. Kreutz (2014, 2021) mentions that Fauré-Fremiet and Penard still assumed a lateral arrangement of the tentacles while Kahl describes the origin of the tentacles ventrally and dorsally from a notch-shaped depression, which also surrounds the rear end of the ciliate. However,

as we have described in our findings, we have observed all the different tentacle arrangements described by all these authors, arrangements that are primarily the result of their state of the tentacles contraction (Fig. 1,2).

#### A. Jankowski

In our understanding, Jankowski did not observe any of the three members of the genus *Legendrea* himself. He published a revision of the genus *Legendrea* in the abstracts of a Young Moldovan Scientists Symposium in 1967 (Jankowski 1967). In that abstract, he created two new genera by dividing the genus *Legendrea* into *Lacerus* and *Thysanomorpha*, respectively, and moved *Legendrea bellerophon* under the new genus *Thysanomorpha*, with the description of the genus not more than what follows; “body edge/body surface serrated, uneven (n.a. = not smooth) with a series of outgrowths with trichomes”. According to the International Code of Zoological Nomenclature these new names are not valid since conference abstracts do not constitute a public and permanent scientific record unless a disclaimer is included (which is not the case). However, these new genera appear accepted in Curds (1982), Corliss (1979) and in Aescht (2001). In 2007 Jankowski published these genera once again in a Russian book (Jankowski 2007) validating the genera, without any drawings. Moreover, the criteria for separation of these different genera are not given in Jankowski (2007) and, consequently, the justification for the erection of those genera remains unclear. However, based on Jankowski (1967), the location of the papillae with extrusomes seems to be the reason. Jankowski uses the term “toxophore” to describe the finger-like appendages, whether they are long and tentacle-like in *L. loyezae*'s case or short and stubby in *Lacerus*' case. Then he separated the genera based on the position of these so called “toxophores” on the cell. Thus, if they are on the posterior end, the cells were assigned to the genus *Legendrea*; if they were placed in the circumference of the cell in a row and extendable, then the cells were assigned to the genus *Thysanomorpha*; finally, if they were in a row but circumference the cell halfway, the cells were assigned to the genus *Lacerus*. The separation was done without thoroughly examining Penard’s and Kahl’s remarks on

the two species *L. bellerophon* and *L. loyezae*, hence missing two of the legendary authors' mentions of the irregular position of the tentacles in *Legendrea loyezae*.

Our documentation of the controlled movement of the tentacles in *L. loyezae*, as well as the variable position of them on the cell due to the plasticity of the organism makes the validity of genus *Thysanomorpha* highly questionable. As we show in this study, *Thysanomorpha bellerophon* and *Legendrea loyezae* not only share close morphology but also show the behaviour of extension and retraction of the tentacles, which may suggest a close phylogenetic relationship. Until future genomic data become available, at the morphological level *Thysanomorpha bellerophon* should be relocated back under *Legendrea*, thus becoming *Legendrea bellerophon*, as originally described by Penard (1914).

### Phylogenetic Analysis

Phylogenetic analysis of the 18S rRNA gene sequence from *L. loyezae* (Fig. 4) firmly places *L. loyezae* within the Family Spathidiidae, Order Haptorida. It is important to note that we were only able to obtain a shorter 18S rRNA sequence for *L. loyezae* and thus its exact placement within the Haptorida needs to be carefully interpreted and would likely change if the complete 18S rRNA gene were to be sequenced. With the shorter sequence of *L. loyezae* (1007 bp) this ciliate forms a sister group (bootstrap support = 38) to sequences from *Epispathidium papilliferum* and an unidentified *Epispathidium* sp. Interestingly, *E. papilliferum* is a spathidiid that bears protruding papillae with extrusomes that are reminiscent of the papillae in *L. loyezae*; however, the papillae in *E. papilliferum* are present in the oral region only.

*L. loyezae* is one of the most interesting organisms we have ever come across either in situ or in literature, not just because of its strange morphology but because of its remarkable behaviour. Our discovery of the controlled tentacle extension after 114 years of the species' first description proves the importance of microscopy and the value of descriptive biology once again. There still are many ciliate species out there lacking the benefit of any microscopical examination. We hope that the study here presented inspires the readership to spend more time looking through a microscope and

reminding them that science is done to subside the mind's curiosity, not incite discord in one's rather short lifespan.

### Methods

**Sampling locations:** Samples were collected from two woodland freshwater lakes in Warsaw (Poland): Las Kabacki (coordinates 52.1254794, 21.0448959) and Zielonka (coordinates 52.3178718, 21.1681360). The study sites are 25 km from each other.

**Sample collection:** Sampling was done by using a telescopic rod with an attached plastic bottle at the tip. The bottle was dipped into the water column mouth looking down and kept in this position until the bottom of the pond was felt, later the sapropel was collected by rotating the bottle above the sediment surface. Therefore, the sapropel was sucked into the collection bottle by the vacuum that formed due to the escaping air. Collected samples were kept in the dark and at room temperature in tight shut containers to avoid oxygen diffusion.

**Microscopy:** Samples were checked daily, often 12 hours a day, some of the samples were examined regularly for months. All microscopy observations were done by using a Zeiss Axioscope 5 with bright field, Differential Interference Contrast, and Fluorescence Microscopy. Zeiss Neofluar 10x 0.3NA, Zeiss Neofluar 20x 0.50NA, Zeiss Neofluar 40x 0.75NA and Zeiss 63x 1.40NA Plan-Apochromat objectives were used.

**DNA extraction, amplification, and sequencing:** Due to the ciliate's rarity, the molecular data were acquired from only a single specimen that was collected from the freshwater sediment of the lake in Las Kabacki (see above). The cell was hand-picked, washed several times with mineral water and transferred into an Eppendorf tube and frozen for molecular analysis. To ensure lysis of the cell, the Eppendorf tube was subjected to 1–5 freeze–thaw cycles (between –80 °C and room temperature), and then MDA protocol using REPLI-g Single Cell Kit according to the protocol was performed. To amplify the 18S rRNA gene PCR amplification was performed with Phusion polymerase (ThermoScientific) and with primers covering the region V4–V9 of 18S rDNA: Euk528F: 5'- CGGTAATTC-CAGCTCC and U1492R and 5'- GGTTACCTTGTTACGACTT (Edgcomb et al. 2011). VProducts were cleaned with a Syngen DNA clean-up Kit and sequenced with Sanger sequencing at the external company Genomed S.A. (Poland).

**Phylogenetic analysis:** Candidate sequences for the phylogenetic tree were downloaded from the Genbank NCBI. Sequences were aligned using ClustalW (Thomson et al. 1994) and trimmed in BioEdit (Hall 1999). The substitution model was determined using MEGA 11 (Tamura et al. 2021) and the phylogeny was created using 1000 iterations of the Maximum Likelihood method and Tamura 3-parameter model G + I (Tamura 1992). A total of 50 sequences have been used, with *Amphileptus aeschtae*, *Litonotus paracygnus*, *Loxophyllum rostratum* and *L. jini* as outgroups. After trimming, 1007 nucleotide positions were compared.

### CRedit authorship contribution statement

**James Weiss:** Conceptualization, Investigation, Resources, Visualization, Writing – original draft, Writing – review & editing. **Demetra Andreou:** Software, Validation, Writing – review & editing.

**Genoveva F. Esteban:** Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing.

#### Data availability

Data will be made available on request.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary Material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.protis.2022.125912>.

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