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Evolution of sperm morphology in *Daphnia*

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Abstract

Sperm, the male most fundamental reproductive feature allowing egg fertilization, evolves under sexual selection. Two components of sperm are mainly under selection, their number and their morphology (including the associated quality). Here we study the evolution of sperm morphology in the genus *Daphnia*. Based on microscopic observations of sperm morphologies mapped on a *Daphnia* phylogeny, we found that increase in sperm length evolved at least twice in *Daphnia*, once in the *Daphnia sensus lato* clade and once in the *Ctenodaphnia* clade. Furthermore, *Daphnia s.l.* lost the ability of cell compaction by losing extracellular encapsulation and exposing large filaments. We discuss the potential reasons for such convergent evolution in sperm morphology.

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Introduction

Sexual selection is a form of natural selection acting on mating and fertilization success. Hence, sperm, the most fundamental male reproductive feature allowing egg fertilization, evolve—at least in part—under such selection. Two components of sperm are mainly under selection, their number and their morphology, the later including the associated quality. Males generally release many gametes, the ejaculate, to fertilize a few eggs. The ejaculate size is known to evolve in response to the risk of sperm competition for egg fertilization, to cryptic female choice and to female receptacle size (i.e. the dilution effect) (Roldan, 2019). In contrast, while sperm are considered as one of the most taxonomically diverse and rapidly evolving cell types (Birkhead *et al.*, 2009; Ramm *et al.*, 2014), the understanding of the adaptive value of sperm morphology, such as length and shape, remains largely incomplete (Lüpold & Pitnick, 2018). Sperm length does not necessarily correlate with increased swimming speed (although it is often the case, see (Tourmente *et al.*, 2011; Rowley *et al.*, 2019)) and structures not involved in velocity evolve most probably in response to the environment in which fertilization occurs but the details are rarely, if ever, clear (Lüpold & Pitnick, 2018).

Daphnia are crustaceans reproducing by cyclical parthenogenesis and as such, egg fertilization is sporadic, but essential for diapause in freezing and drying habitats and for dispersal. Following periods of clonal reproduction, during which females only produce genetically identical daughters, and usually triggered by a change in environmental conditions, some females produce sexual eggs while others produce males. During mating, generally one male, but sometimes more, attach to the female to fertilize eggs which will be laid into the female brood pouch after the male(s) departed (Duneau *et al* in prep.). The brood pouch is a receptacle formed by the carapace and present on the dorsal side of all *Daphnia* species receiving either clonal or sexual eggs. For the latter, the cuticular structure of the brood pouch changes to form a protective case which will be released upon molting, creating genetically diverse egg-banks from which future populations can be established. Fertilization takes most likely place in this brood pouch (Duneau *et al.* in prep.). However, although there are recordings of males competing for fertilization in *Daphnia magna* (Duneau *et al.* in prep.), the extend of sperm competition in this receptacle is unknown. Importantly, a water current generated by the filtering

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apparatus oxygenates the eggs in the brood pouch (Seidl *et al.*, 2002), and it is likely that many sperm may be flushed out after the male ejaculated. In this context, males are expected to evolve larger ejaculates. However, assuming finite resources allocated to sperm production, this may come at the cost of sperm length (Immler *et al.*, 2011), an important trait in male competition. Thus, males are likely to evolve persistence traits that allow them to increase the chances of fertilization. Here we investigate how sperm morphology in *Daphnia* species diverged in a phylogenetic context, presumably as a response to sexual selection.

Although pioneer studies have given key general descriptions to identify the main structures (Delavault & Berard, 1974; Wingstrand, 1978; Zaffagnini, 1987; Wuerz *et al.*, 2017), only little is known about sperm morphology in *Daphnia*. All *Anomopoda*, an infraorder including *Daphnia*, have a vacuolar type of spermatogenesis (Wingstrand, 1978), *i.e.* in the testes, the spermatids are enclosed in "private" vacuoles in the nutritive cells and are exocytosed into the testicular lumen after they have decreased strongly in size and matured. After they have compacted, they are generally small, about a few microns. Sperm of *D. magna* has been more thoroughly studied with recent technology. This *Ctenodaphnia* species has larger sperm (~10 µm) encapsulated by an acellular capsule likely compacting radial arms probably to pack more sperm in the testes (Wuerz *et al.*, 2017). The roles of the capsule and of the filaments that are only visible surrounding the cell within the vacuole with electronic microscopy (Wuerz *et al.*, 2017) are unclear. Based on comparison with other models, it has been proposed that female secretion could dissolve the capsule and the filament could have a role in the fusion between gametes (Wuerz *et al.*, 2017). Here, we used a robust phylogeny of the Daphnidae (Adamowicz *et al.*, 2009; Cornetti *et al.*, 2019) and assessed several species representing major clades within *Daphnia* to better understand the evolution of sperm morphology in this genus.

Materials and methods

Male *Daphnia* were either sampled from female mass cultures in the laboratory, where males are naturally produced as a consequence of high density, or from females exposed to the hormone methyl farnesoate (MF, 40nM final concentration) to induce male production. We induced male

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production for *D. hyalina*, *D. zschokkei*, *D. mendotea*, *D. galeata*, *D. curvirostris* and *D. dentifera* and collected naturally produced males for *D. similis*, *D. sinensis*, *D. lumholtzi*, *D. carinata*, *D. magna*, *D. hispanica*, *D. dolichocephala*, *D. barbata*, *D. longispina*, *D. pulex* and *D. pulicaria*. Note that *D. hyalina* and *D. zschokkei* are now synonymous of *D. longispina* and should be understood as *D. longispina 'hyalina'* and *D. longispina 'zschokkei'* (Petrusek *et al.*, 2008), hence we merged them on the same branch in the cladogram.

To collect sperm, we exposed mature males to a 1 % nicotine solution ((-)-Nicotin 162.23 g/mol, from Carl Roth, Germany) to induce ejaculation as in (Duneau *et al.*, 2012). As only mature spermatozoa are in the testicular lumen (p11 in Wingstrand, 1978; p277 in Zaffagnini, 1987), this method allowed us to describe and measure mature sperm and avoid immature ones. Presence of filaments on the sperm was recorded, but we did not measure their length. Measurements of the longest length of the sperm were performed with ImageJ (v. 1.5i) using photographs taken under phase contrast light at magnification 40x. In species with very small sperm (*D. pulex*, *D. pulicaria*, *D. dolichocephala* and *D. barbata*) we paid particularly attention that the sperm were just released from the spermiduct to reduce the possibility of degradation or to confuse them with other particles. However, it was challenging to take photographs of them, and the measurement may be less accurate than for the other species. *D. pulex* sperm length is only around 2 µm in length (Xu *et al.*, 2015). All sperm were also observed at the moment of release from the ejaculatory opening to verify that their shape corresponds to what was observed later when they settled and were photographed. We also observed sperm morphology in sea water to confirm that osmolarity was not affecting our results. Drawing of male abdomens with the genital papilla were taken from published keys (Benzie, 2005; Popova *et al.*, 2016).

Results

Sperm morphology varied greatly among *Daphnia*, ranging from about 2 µm to at least 20 µm (Figure 1 and supplementary figures 1 and 2). There was a clear phylogenetic signal in sperm length across *Daphnia*, but length clusters are polyphyletic. Based on recent *Daphnia* phylogenies (Adamowicz *et al.*, 2009; Cornetti *et al.*, 2019) and the microscopic observations of sperm

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morphologies, we found that an increase in sperm length evolved at least twice in *Daphnia* (Figure 1), once in *Daphnia sensus lato* and once in *Ctenodaphnia*. This length variation was probably mostly due to a difference in sperm compaction by an extracellular capsule. We found that clades leading to the *D. longispina* group have lost entirely this capsule, hence leaving long filaments without protection (Figure 2, Supplementary figures 1, 2 and 3).

Ceriodaphnia, our outgroup, have sperm of the vacuolar spermatogenesis type, like *Daphnia* species and the rest of the infraorder of the *Anomopoda*. Their sperm have been described, based on electron microscopy, as small (2 to 6 µm), more or less rod-shaped and strongly compacted in their capsule (Figure 2A and p25-26 in Wingstrand, 1978). This information based on several *Ceriodaphnia* species allowed to determine the most parsimonious ancestral *Daphnia* sperm morphology. The *Ctenodaphnia* group, except for *D. dolichocephala* and *D. barbata* who had compacted and small sperm, evolved non-compacted and elongated sperm, several times larger than the ancestral morphology (Figure 1 and 2B). A similar adaptation occurred in *Daphnia s. l.*. As in (Xu *et al.*, 2015) which reported *Daphnia pulex s. str.* sperm length, *Daphnia* from the *D. pulex* subgroup conserved the small and compacted sperm morphology, while *D. curvirostris* and sister species from the *D. longispina* species complex also evolved larger elongated sperm, but not as large as in *Ctenodaphnia* (Figure 1). Additionally, it seems, that *Daphnia* species from the *D. longispina* species complex lost the extracellular capsule compacting the sperm and have long filaments with potentially forked structures (Figure 2C). These filaments can be several times the length of the sperm (not measured here) (Figure 2 and Supplementary figure 3). In the same ejaculate from a single *D. longispina s. str.* male sperm can have, on each side, either one long filament or many shorter ones (see Figure 2 and Supp. material). It has been argued that ejaculate size increases with female receptacle length to compensate for the dilution effect and that it should be accompanied by a reduction in sperm length. Considering that most *Ctenodaphnia* are not much larger than *Daphnia s. l.*, it is unlikely that this hypothesis explains the evolution of sperm morphology in *Daphnia*.

We further compared the shape of the male genital papilla across the *Daphnia* species, using drawing from published keys (Benzie, 2005; Popova *et al.*, 2016) (Figure 1). Most species have no or a very

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inconspicuous papilla, the biggest exception is the papilla structure present in *D. magna* (Figure 1). This structure is also found in several species related to *D. magna*, such as *D. atkinsoni*, *D. bolivari* and *D. mediterranea*, but they are not included in our study (Flössner, 2000; Benzie, 2005).

Discussion

Our assessment of sperm morphology uncovered clearly structured phylogenetic variation in sperm length and in the presence or absence of long filaments. The reason for this variation is unclear but may be in part explained with the strength and intensity of post-copulatory sexual selection. Strength of sexual selection is a function of how often male ejaculates compete for fertilization, in particular direct sperm competition and sperm competition through cryptic female choice.

The frequency of sex in *Daphnia* depends on the species and of the environment, being eventually under local adaptation (Roulin *et al.*, 2013). In unstable and short-lived habitats, such as small rockpools or ponds in unstable or strongly seasonal environments, such as deserts and arctic sites, few asexual generations occur before diapause recommences. In stable environments, such as large lakes and ponds in temperate mild climatic regions, many asexual generations may occur before the next sexual generation comes, if it ever comes in a mother's lifetime. Traditionally, the later type received more attention by *Daphnia* researchers, leading to the wrong impression that sexual reproduction, and thus the occurrence of males, is generally rare. Therefore, it is difficult to estimate the intensity and frequency of sexual selection in the system, especially for each species. In certain populations, males can be periodically abundant, and several males can be found copulating at the same time with a female (Duneau et al, in prep). It is not clear whether fertilization would occur in the brood pouch or in the oviduct. However, the fact that most genital papilla are inconspicuous suggest that fertilization is realized in the brood pouch as the access to the oviduct seems complicated. Thus, cryptic female choice and male-male competition via sperm competition, both likely to shape the evolution of sperm morphology, may occur in this female receptacle.

By ejaculating in the female receptacle, males face the challenge to have their sperm flushed out, a phenomenon which could be a form of cryptic female choice. To provide oxygen, the brood pouch is

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continuously flushed with a stream of water, entering from the caudal end and leaving from the ventral carapace chamber in a pulsed manner resulting from the rhythmical movements of the limbs (Seidl *et al.*, 2002). This stream might flush out sperm. It is likely that this mechanism selects for males able to produce a large number of sperm of good quality. Interspecies variations in such cryptic female choice may or not vary among species, but males in each species may have different features to increase their chance to be chosen. Sperm may attach and cover the inner lining of the brood pouch, so eggs would not be expelled and touch the sperm as soon as they are released in the brood pouch. However, there is no obvious structure supporting that freshly ejaculated sperm can attach to the inner lining. Then, they may simply increase the number of sperm per ejaculate to increase the chance for few sperm to remain in the brood pouch. However, as most sperm production is done early in male's life (Wuerz *et al.*, 2017) the total number of sperm is limited by the size of the spermiduct. By limiting the total number of sperm stored, this constraint may put selection on the amount of compaction allowing to store more cells in the duct. In *Daphnia*, there is an extracellular compaction process by an extracellular vacuole before the mature sperm is released into the spermiduct (Wingstrand, 1978). We propose that the evolutionary changes in sperm length we observed may in part be due to a change in the mechanism of cell compaction before maturation.

Female cryptic choice may select for higher number hence smaller sperm, but sperm of different males may also compete within the receptacle imposing an additional selection pressure on sperm morphology. Such sexual selection on sperm morphology through male-male competition will increase as males are more numerous at a given time and regularly mate at the same time with a female. The change in sperm size as a result of sperm competition is a commonly seen evolutionary pattern (see for an example Vielle *et al.* (2016), including extreme examples like giant *Drosophila* sperm (Lüpold & Pitnick, 2018). However, it is difficult to assess the intensity of sperm competition based solely on sperm size. In *Caenorhabditis elegans*, experimentally enhanced sperm competition leads to the evolution of larger sperm (LaMunyon & Ward, 2002). However, in *Drosophila melanogaster*, the competitive advantage was present only in females with relatively long sperm storage organs (Miller & Pitnick, 2003). When sperm are non-motile, the outcome of sperm

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competition generally resembles a lottery in which having more tickets than your competitor increases your chances to win, males are thus expected to invest in sperm production. Assuming finite resources allocated to sperm production, this may then come at the cost of sperm size (Immler *et al.*, 2011). When sperm competition is high, the total number of sperm may be maximized balancing the size of the sperm *per se* and its compaction. However, when sperm competition is low, males may invest less in sperm size and there may be little benefit from expending energy in mechanisms insuring constant sperm length (Bauer & Breed, 2006; Immler *et al.*, 2008). However, because it often correlates with velocity, increase in sperm size can also be selected if sperm are motile at some point of the fertilization process. It is difficult to assure that *Daphnia* sperm are always non motile. Sperm in the brood pouch are probably not motile but we observed them on a microscope slide and an *in vivo* assessment would be necessary to ascertain sperm behavior (Lüpold & Pitnick, 2018). *Daphnia* males ejaculate into the brood pouch filled with water (the same used for our observation); it is therefore likely that the absence of motility observed in water on a microscope slide reflects what is happening in the brood pouch. However, it has been shown that fluid surrounding eggs of externally fertilized species induced modification to sperm motility (Yoshida *et al.*, 2013). In such scenario, the capsule might break, the sperm would attach to the oocyte, crawl to an eventual specific fusion site and larger sperm with filaments may be advantageous in the race to this site.

Our phylogenetic analysis of sperm morphology revealed a monophyletic clade (i.e. *D. longispina* species complex) with sperm exposing filaments of diverse shape and length. In *Daphnia magna*, it seems that filaments exist and are inside the extracellular vacuole (Wuerz *et al.*, 2017), but they are difficult to resolve even with electron microscopy and therefore our knowledge about their ultrastructure is limited. Nevertheless, the vacuole is expected to break before the sperm cell fuse with the oocyte and filaments to be exposed. *Daphnia* species from the *D. longispina* species complex evolved non-compacted sperm and the apparent loss of the capsule exposes especially long, sometimes numerous, filaments. Those exaggerated long structures are unlikely to be flagella and not used to move towards the eggs as they are extremely flexible and can be forked. But, as sperm features are expected to be adaptations to their specific fertilization environment (Pitnick *et al.*, 2009), filaments

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almost certainly have a role in fertilization. If we cannot not exclude that they have a role in attachment to the brood pouch to avoid being flushed out with the water flow generated by the female, it is very likely that they have a role in the fusion with the oocyte and potentially with crawling at its surface to reach a fusion site and win the eventual sperm competition. It may be that with reduced selection for compaction, readiness to fuse with the oocyte may be favored by the exposure of those long filaments.

As it is often the case when studying the evolution of sperm morphology, it is difficult to clearly identify the role of sperm features. However, it is certainly evolving driven by the intensity of sexual selection in the system. *Daphnia* is a cyclical parthenogenetic species, where periods of sexual reproduction are interspersed with period of asexual reproduction. Cyclical parthenogenetic species, like Daphnia, aphids, and rotifers, alternate sexual and asexual reproduction. The number of asexual generations in between two sexual reproductive events may be highly variable, but this is important because the less frequent sexual reproduction is, the lower the average intensity of selection. Thus, one can wonder how the variation in intensity of sexual selection among cyclical parthenogenetic species influences the evolution in sperm morphology. Here, the missing information on the sexual process in *Daphnia* species makes difficult to assess the relative role of cryptic female choice and sperm competition but both are likely to play a role in the convergent evolution or maybe, even more interestingly, the conserved evolution of sperm length in *Daphnia*. Altogether, our results support the idea that sexual selection plays a role in cyclical parthenogenetic invertebrate species and encourage further investigations.

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Figure legends

Figure 1: Evolution of sperm and genital papilla morphologies in Daphniidae. Phylogeny of Daphniidea is modified from Cornetti et al. 2019 and Adamowicz et al. 2009. Purple, pink and green represent respectively the sperm that are small, large with capsule or large with filaments exposed. Drawings represent the genital papilla of the males (from Benzie 2005 and Popova et al. 2016) and indicate the atypical exaggerated structure of *D. magna*. Scale bar represent 0,1mm. Photographs show an example of sperm for each species. The graph represents the difference in sperm length among males. The mean sperm length was calculated with 2 to 3 ejaculates and is intended to provide a rough sense of the traits.

Figure 2: Examples of Daphnia sperm morphologies. A- *Ceriodaphnia laticauda*, ancestral small and encapsulated elongated sperm (purple color in figure 1). Photo from (Wingstrand, 1978). B- *D. magna*, large and encapsulated elongated sperm of the groups (salmon color in figure 1); C and D - *D. longispina*, two examples of typical sperm non encapsulated and elongated sperm with filaments (green color in figure 1).

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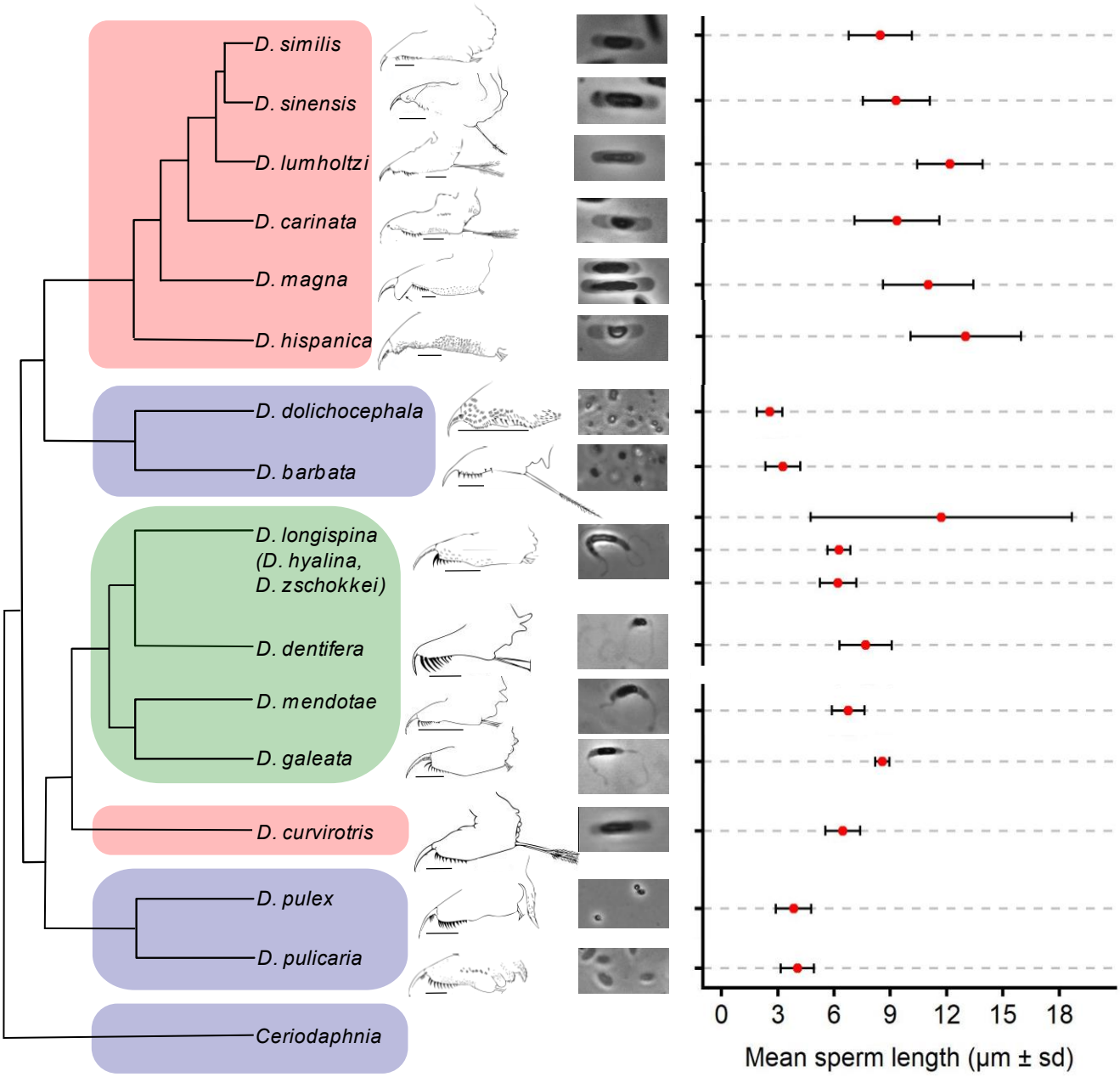

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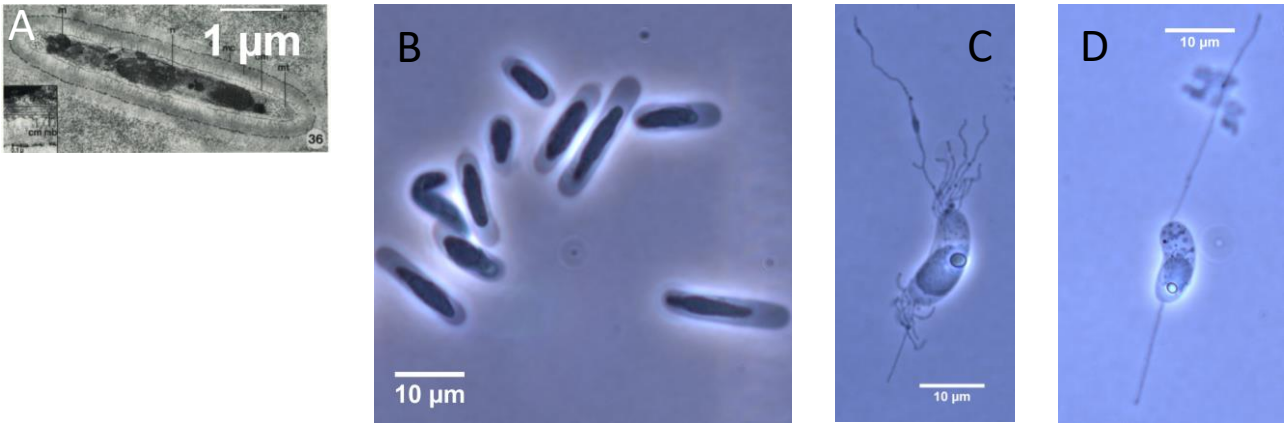

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