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Review Article

Future prospects for investigating ciliate biodiversity

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Abstract. Ciliates have a long history of being central in evolutionary and ecological studies on eukaryotic microorganisms. Although thousands of species have been discovered, their total diversity still remains unknown. Here, we will discuss two unsolved problems that hinder the further exploration of ciliate diversity at the species level, and potential solutions to these problems are proposed. First, ciliate morphospecies are difficult to identify because the different silver stains are not scalable (they do not represent high-throughput methods) and basic supplies are lacking (e.g., protargol); a solution may be the development of fluorescent staining techniques. Second, ciliate phylogenetic species are difficult to identify because of extensive paralogy in nuclear-protein-coding genes; a solution may be to concentrate on sequencing mitochondrial genomes. These two approaches could be integrated into a high-throughput fluorescent-single-cell sorting and mitochondrial genomes sequencing process that would enable the observation and better understanding of ciliate species on a massive scale.

Keywords: Biodiversity, Ciliophora, fluorescence, staining methods

INTRODUCTION

Correct species identification is crucial for understanding the ecology and evolution of any group of organisms. Species are often used as the fundamental unit in many ecological and evolutionary analyses and such comparisons are routinely performed with ciliates. Observing ciliate species has a long history (Dobell 1932, Lynn 2008), and although ciliates are microbes, their cells are relatively large compared to other eukaryotic microbes and have complex morphological characteristics (Dunthorn and Katz 2008). Thanks to this, scientists have been able to identify and describe ciliates into species since the beginning of their observation using morphological methods (e.g., Kahl 1931) till nowadays (Dunthorn and Katz 2008, Lynn 2008, Foissner 2014, Warren *et al.* 2017, Abraham *et al.* 2019). Ciliates also have been extensively studied in molecular phylogenetic studies using various molecular markers (Dunthorn *et al.* 2014b, Gao *et al.* 2016, Fernandes and Schrago 2019), and in more recent years by phylogenomic methods (Gentekaki *et al.* 2017, Lynn *et al.* 2018, Lasek-Nesselquist and Johnson 2019, Rotterová *et al.* 2020). Here we discuss morphologic and genetic methods that

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might complement currently used techniques for identifying ciliate species now or in the near future.

CILIATE MORPHOSPECIES

Advantages and disadvantages of classical silver staining methods

Silver staining techniques are the standard way to uncover ciliate morphological features (Lynn 2008). These techniques are beneficial as they stain structures that are not visible or difficult to study by live observation: the arrangement of kinetosomes in the somatic and oral ciliature and their associated fibers, as well as the margins of the alveoli (known as silverline system or argyrome) (Foissner 2014, Warren *et al.* 2017, Abraham *et al.* 2019). Depending on the technique, silver staining techniques might also visualize further diagnostic features such as the nuclear apparatus, extrusomes, nematodesmata (microtubules originating at the proximal end of the kinetosomes), the cytoproct, and myonemes (Lynn 2008, Foissner 2014).

The silver staining methods applied in ciliatology can be classified into three groups by the chemicals used (Foissner 2014, Abraham *et al.* 2019). (i) Silver nitrate stains, the pioneer ciliate staining inspired by techniques used in neurohistology and later modified into "wet" and "dry" silver nitrate stains. They reveal the silverline system and often provide good results for staining the infraciliature. However, the "dry" silver nitrate method does not sufficiently work for marine/brackish ciliates (Abraham *et al.* 2019), and "wet" silver nitrate methods are complicated and do not allowed for proper characterisation of the nuclear apparatus (Chatton and Lwoff 1930, Kirby 1945, Wilbert 1975, Lynn *et al.* 1981, Foissner 2014).

(ii) Silver carbonate stain, a rapid and relatively simple silver staining method that is based on ammoniacal silver carbonate solution and organic pyridine. Silver carbonate methods reveal the infraciliature and the nuclear apparatus. However, this method produces short-live preparations, requiring an investigation within the following hours (Fernandez-Galiano 1976, Augustin *et al.* 1984). The method is also not ideal for observing the argyrome (system of argentophilic membrane structures) and does not work well for some ciliate groups, such as the Hypotrichida (Foissner 2014).

(iii) Protargol stain, the most used staining method in ciliate taxonomy. This stain is based on silver proteinate with the brand name Protargol that is currently no longer commercially available and has to be synthesized (Pan *et al.* 2013). The protargol methods reveal many cell structures such as basal bodies, cilia, and the nuclear apparatus, but are not suitable to reveal the argyrome, and is not useful for uncovering the infraciliature for certain groups of ciliates such as *Paramecium* (Foissner 2014).

Multiple problems are associated with silver staining methods. One of the prevailing concerns is that none of these methods are equally effective for all ciliate groups, and none reveals all the morphological characters necessary for taxon delineation (Foissner 2014, Abraham *et al.* 2019). In other words, there is no universal silver staining technique to follow, but several methods with numerous protocols that one needs to combine for species identification depending on the ciliate taxon or morphological structure of interest. Another general problem is that multiple specimens have to be isolated to make successful stainings, since many cells are inevitably contracted, swollen, or broken after the fixation or bleaching process. This rich culture requirement is a drawback for the low abundant taxa, and the majority of taxa occurs with low to very low abundances (Dunthorn *et al.* 2014c). Moreover, some ciliate groups cannot be sufficiently stained at all, such as members of the genera *Pseudoprorodon* and *Cyrtolophosis*, and most free-living litostomateans (Abraham *et al.* 2019). In addition, hazardous substances, such as osmium tetroxide solution (OsO_4) are used for a fixation of many taxa by several silver staining methods (Chatton and Lwoff 1930, Foissner 2014).

The abovementioned silver staining methods are used for qualitative studies, such as species descriptions. Montagnes and Lynn (1987) established a quantitative protargol staining protocol to enumerate and identify ciliates in community analyses. This method is applied and improved in various, mainly ecological, studies (Skibbe 1994, Pfister *et al.* 1999, Wickham *et al.* 2000, Acosta-Mercado and Lynn 2003, Van Wichelen *et al.* 2013, Medina *et al.* 2016, Zhao and Xu 2016, Pitsch *et al.* 2019, Yang *et al.* 2020). Although quantitative protargol stain visualizes ciliate taxonomic characteristics, the quality is often inferior to the qualitative methods. Also, some fragile species may rupture during the preparation procedure (Montagnes and Lynn 1987, Skibbe 1994, Pfister *et al.* 1999). Depending of the staining technique applied, it is occasionally challenging to correlate the stained specimens to their living morphology without an *in vivo* pre-observation.

Besides the silver staining techniques, two fluorescent staining methods have been proposed to visualize some of the diagnostic structures in ciliates but have never been fully implemented in ciliate diversity or taxonomy research (Abraham *et al.* 2019). The first one is taxoid staining, a fast method for uncovering the arrangement of kinetosomes (Arregui *et al.* 2002, 2003, Kovács and Csaba 2006, Barasoain *et al.* 2010). The second one is immunofluorescence staining, a precise method for visualization of the infraciliature (Jeanmaire-Wolf *et al.* 1993, Aubusson-Fleury *et al.* 2015). The potential of both approaches in ciliate research is discussed below.

Taxoid staining, a promising future approach

To overcome the complexity of traditional silver staining techniques that are time-consuming and laborious, we advocate for the development of a fluorescent method based on the Arregui *et al.* (2002, 2003) approach. The method is based on a fluorescent taxoid (derivate of taxol, a natural product produced by the Pacific Yew, *Taxus brevifolia*) that binds to microtubules, and a fluorescent DNA stain. In this way, some of the ciliate species-specific features such as the ciliary pattern and the nuclear apparatus can be revealed. This was already tested on eight ciliate species (*Chilodonella uncinata*, *Coleps hirtus*, *Colpidium campylum*, *Euplotes focardii*, *Paramecium tetraurelia*, *Sterkiella cavicola*, *Tetrahymena thermophila*, *T. pyriformis*) (Arregui *et al.* 2002, 2003, Kovács and Csaba 2006, Barasoain *et al.* 2010). We think, this method can be improved by applying a deciliation (removing cilia) step, as cilia around the cell attract taxoids and hinder direct observation of the subjacent kinetosomes. The use of simple fluorescent stains would keep the process relatively inexpensive and not too time consuming.

The proposed theoretical taxoid staining approach comprises three steps. The first step is the deciliation that enables the observation of the uncovered kinetosomes and could be performed using liquid detergent (Foissner 2014), manganese (Cohen *et al.* 1982), chloral hydrate (Nelson 1995), or ethanol and calcium (Aubusson-Fleury *et al.* 2015). Although none of the methods removes all cilia from all specimens, the best results were obtained using the ethanol and calcium method (Aubusson-Fleury *et al.* 2015). As oral grooves, such as those in *Paramecium*, somehow protect the cilia against deciliation, there is still room to improve the deciliation procedure to become equally effective for all ciliate taxa regardless of their cell shapes.

The second step is applying taxoid dyes to visualize the species-specific ciliary patterns. Although several fluorescence compounds may be used to stain microtubules (see Aubusson-Fleury *et al.* 2017), taxoid dyes are convenient as they strongly and directly bind to microtubules without using antibodies (Lynn and Small 1981, Abal *et al.* 2001). Another advantage of taxoids is that the previous fixation and permeabilization processes are not necessary, and the whole procedure takes a few minutes (Lecke and Tasca 2002, Barasoain *et al.* 2010, Abraham *et al.* 2019). Also, the cell shape and size seem to be unaffected by the taxoid dyes, but this has not been thoroughly tested yet (Barasoain *et al.* 2010). So far, only Flutax-1 and Flutax-2 have been used in ciliates, but other taxoid dyes, such as Hexaflutax, Rotax, and FChitax-3, can also be applied (Barasoain *et al.* 2010).

The third step represents applying a strong DNA-binding stain, such as DAPI (4′,6-diamidino-2-phenylindole), to visualize the number, size, and shape of the micronuclei and macronuclei (Kapuscinski 1995). This provides additional diagnostic features of ciliates. The advantage of using DAPI staining is that it is already established and widely used in fluorescence microscopy and was also successfully applied on ciliates (Lessard *et al.* 1996, Wancura *et al.* 2018, Abraham *et al.* 2019).

The fluorescent methods' major drawback is the lack of permanent slides, as all these dyes fade away over time (Stockert and Blazquez-Castro 2017). Hence, they cannot be used for the (re)description of ciliate taxa according to the current version of the Code that exclusively requires physical specimens (ICZN 1999). Permanent slides are also frequently deposited as voucher material in ecological studies. There is an effort to expand the methodology with novel molecular and morphological approaches (Corliss 1995; Aescht 2001, 2008; Foissner *et al.* 2002; Adl *et al.* 2007; Lynn and Simpson 2009; Santoferrara *et al.* 2016; Warren *et al.* 2017) and elevate classical ciliate taxonomy into modern integrative taxonomy (Dayrat 2005, McManus and Katz 2009, Morard *et al.* 2016, Clamp and Lynn 2017, Vďačný 2017). Introducing fluorescence visualization methods that could be combined with single-cell sorting, metagenomic approaches and environmental sequence data in one unified pipeline may play a vital role in this transformation.

Immunofluorescence methods and possible future applications

Immunofluorescence microscopy methods use fluorophore-tagged antibodies to visualize specific proteins

in cells or tissues. The methods are categorized as: (i) direct, using only primary antibodies; or (ii) indirect, using secondary antibodies that bind to primary ones enhancing the signal (for details, see Im *et al.* 2019). Most protocols comprise three preparation steps: fixation of the cells, epitope/antigen retrieval that removes methylene bridges from antigenic sites, and a blocking step that prevents false signals. Subsequently, the target proteins are labelled by an antigen-antibody reaction (Im *et al.* 2019).

Immunofluorescence microscopy has played a crucial role in numerous cytological studies on the ciliate's cytoskeleton (e.g., Jeanmaire-Wolf *et al.* 1993, Beisson *et al.* 2001, Aubusson-Fleury *et al.* 2013), cell division (e.g., Keryer *et al.* 1987, Iftode *et al.* 1989, Kaczanowska *et al.* 1996), or stomatogenesis (e.g., Keryer *et al.* 1990, Sperling *et al.* 1991, Iftode *et al.* 1997). In many of those studies, basal bodies were visualized using tagging of various specific proteins. The same antibodies and protocols could be employed to visualize the ciliary pattern as it consists of individual basal bodies. To visualize basal bodies, and thus the ciliary pattern, we can target actin (Hauser *et al.* 1980, Iftode *et al.* 1989), centrin (Ruiz *et al.* 2005, Jerka-Dziadosz *et al.* 2013, Aubusson-Fleury *et al.* 2017), or tubulins (Callen *et al.* 1994; Ruiz *et al.* 1999, 2004; de Loubresse *et al.* 2001; Dupuis-Williams *et al.* 2002; Aubusson-Fleury *et al.* 2013). Sometimes, basal bodies are also targeted by proteins such as cytokeratin (Lai and Ng 1991), tetrin (McLaughlin and Buhse 2004), vimentin (Mohr *et al.* 1990), a centrosomal protein FOR20 (Aubusson-Fleury *et al.* 2012), or microtubule-associated proteins such as Bld-10 and Sas-6 (Jerka-Dziadosz *et al.* 2010).

Although antibodies are routinely used in cytological studies of ciliates, they are not applied in ecological or taxonomical research. The reasons for omitting the use of antibodies in ciliate taxonomy include seemingly complex immunofluorescence protocols as well as the fact that these methods do not yield permanent slides. To overcome the complexity, we suggest to implement some of the routinely used antibodies and straightforward immunofluorescence protocols. For example, tubulin antibodies are often used to label basal bodies, and numerous of them are commercially available (see Table 1 in Aubusson-Fleury *et al.* 2015). A potential pilot experiment might use universal α-tubulin antibodies such as DM1A (anti-α-tubulin antibody) that is routinely employed in cytological studies and also available in several life-science companies for a reasonable price. Proteins of the centrin family could be also targeted to visualize ciliate basal bodies using the anti-Centrin 2 antibody as demonstrated in Aubusson-Fleury *et al.* (2017).

Most of the recent ciliate immunofluorescence studies follow the protocol from Beisson *et al.* (2010) for visualizing different cortical structures (including basal bodies) of *Paramecium*. It contains a permeabilization step using Triton-X solution, a fixation in paraformaldehyde, a washing step, a reaction with primary antibodies following incubation and second reaction with secondary antibodies (if used), and the mounting with Citifluor. This protocol is straightforward without using advanced equipment and could be, in theory, the right candidate for ecological studies. However, several problems associated with the use of environmental samples may arise. Such samples may contain inhibitors or contaminants that will hamper antibodies' permeabilization and its binding to the target protein. Therefore, if possible, the environmental samples should be washed several times in distilled water before cell permeabilization or in a cytoskeleton-stabilizing buffer before fixation (Cohen and Beisson 1988).

CILIATE PHYLOGENETIC SPECIES

Advantages and disadvantages of classical molecular methods using nuclear DNA

Molecular approaches to distinguish ciliate species were developed in the early seventies (Nanney 1977). Many of these methods, such as comparison of DNA base ratios (Conner and Koroly 1973), nucleic acid hybridization (Allen and Li 1974), restriction site variation (Din and Engberg 1979), or immunological tests (Loefer and Scherbaum 1963), were shown to be not suitable for species discrimination (Nanney and McCoy 1976). Some techniques such as isoenzymes analysis and randomly amplified polymorphic DNA (RAPD) were commonly used for several decades but were recently replaced by new techniques based on DNA sequencing.

The first widely used sequencing method was Sanger sequencing that provided a direct comparison of the primary DNA sequence (Sanger *et al.* 1977). First studies based on Sanger sequencing were made on *Tetrahymena* species using various molecular markers such as SSU-rRNA (small subunit ribosomal ribonucleic acid) (Sogin *et al.* 1986), LSU-rRNA (large subunit ribosomal ribonucleic acid) (Nanney *et al.* 1989, Preparata

et al. 1989), and histone region (Brunk *et al.* 1990, Sadler and Brunk 1992). Sanger sequencing became a routine tool for obtaining molecular data to analyze the phylogeny that in turn served to establish natural ciliate systematics (Dunthorn *et al.* 2008, Gao *et al.* 2016b, Vďačný and Foissner 2019). The SSU-rRNA became a standard molecular marker in these studies. It contains both conservative and variable regions, allowing one to analyze both ancient and more recent phylogenetic relationships on multiple (from inter- to intraclass) systematic levels. But for ciliate species delimitation, the molecular markers with a higher evolutionary rate proved to be more suited. For example, the internal transcribed spacer region combined with large-subunit rDNA contributed in differentiating morphologically similar taxa in tintinnid ciliates (Xu *et al.* 2012, Santoferrara *et al.* 2015). The internal transcribed spacer region and the hypervariable domains 1 and 2 of the LSU-rRNA indicate endosymbiotic astomate species (Obert and Vďačný 2020), *Spirostomum* species (Shazib *et al.* 2016), as well as *Paramecium* species and their syngens (Stoeck *et al.* 2014).

The next step in the development of molecular methods using nuclear DNA was the introduction of high-throughput sequencing technologies. These sequencing methods combined with DNA barcoding allow to identify not only the target organisms but all presented taxa from the given environmental sample by DNA metabarcoding (for review, see Taberlet *et al.* 2018). DNA metabarcoding facilitates a quick evaluation of the molecular diversity of organisms that are otherwise hard to detect due to their rareness or small size, such as protists (Santoferrara *et al.* 2020), including ciliates (Forster *et al.* 2012, 2015; Gimmler *et al.* 2016; Boscaro *et al.* 2017; Stoeck 2018). To identify a ciliate species presented in an environmental sample, the gained sequences are compared against one of the currently available molecular databases with already identified taxa. Short segments of the SSU-rDNA (e.g., hyper-variable V4 and V9 regions) are mainly used as a barcode sequence (Dunthorn *et al.* 2012b). Because of the short length, barcode sequences are often not informative enough to successfully distinguish all sampled taxa on species level. Therefore, most of the metabarcoding studies use higher classifications such as the class level in downstream analyses (Gimmler *et al.* 2016, Canals *et al.* 2019, Fernandes *et al.* 2021).

Another potential problem is that reference databases may not cover a significant portion of the diversity, for instance, the study of Fernandes *et al.* (2021) on Brazilian Atlantic Forest water bodies, nearly one third of the ciliate taxonomic units shared less than 97% sequence identity to any reference sequences. Likewise, Venter *et al.* (2018) compared high-throughput sequencing and morphological data of soil ciliates and uncovered multiple gene variants for single morphospecies of dominant well-studied ciliate taxa. They proposed that environmental clone sequences of undetermined taxa likely belong to known species that have been described only by morphology. This demonstrates the importance of integrative taxonomy that ties together morphological and molecular data, as shown in Pitsch *et al.* (2019). Furthermore, the number of rDNA copies in ciliates varies greatly (Gong *et al.* 2013), and the rDNA copy number does not correlate with the cell abundance but roughly with the cell biomass (Fu and Gong 2017). This is especially critical in ecological studies aiming to estimate cell abundances or biomasses based on read numbers of rDNA gene sequences. Sequence variation within individual genomes (intragenomic polymorphism) might represent another obstacle for molecular-based species identification. In ciliates, intragenomic polymorphism of the SSU rRNA gene is generally below 3%, yet, it varies between species (Wang *et al.* 2019, Zhao *et al.* 2019). The filtration steps and removal of rare sequences in a bioinformatics pipeline minimize the effect of intragenomic polymorphism.

While nuclear SSU-rRNA genes have been widely used to identify/discriminate ciliate species and to infer their phylogenetic placement, nuclear protein coding gene have been used less often because of the presence of paralogous genes. Paralogs arise during the gene duplications independently from speciation events and therefore do not reflect a species evolution history but rather a gene-family tree (Koonin 2005). The presence of paralogs in ciliates is well-documented for *Paramecium tetraurelia* (Aury *et al.* 2006) and *Tetrahymena thermophila* (Eisen *et al.* 2006). As dealing with paralogs represents a crucial step when using protein genes in molecular analyses, we discuss the current practice and possible solutions for recognizing paralogs in the following paragraphs.

Even though there is no perfect method of recognizing and filtering out of paralogs, Lynn *et al.* (2018) used a thorough procedure to minimize their influence in their phylogenomic analyses. In the first step, the known paralogs are removed based on a reference database (Pruitt *et al.* 2007). Then, every gene is manually inspected in single-gene trees to recognize putative paralogs for every added specimen and each gene. Al-

though this control procedure is arduous for a phylogenomic analysis that contains hundreds of genes, it may prevent biased results. To identify potential paralogs in single-gene trees more precisely, Lynn and Kolisko (2017) distinguished three main types of paralogs. The first is the in-paralog type that originates within a species after a speciation event. In the phylogenetic trees, in-paralogs occur only within a particular species in terminal nodes and not elsewhere. The authors suggest keeping the longest sequence or sequence with the shortest branch if in-paralogs occur. The second is the mid-paralog type that originates in a particular group or subgroup (e.g., in some genera or families in terms of taxonomy). In this case, the authors recommended to keep only sequences belonging to one ortholog group. And finally, the last is the deep-paralog type that represents genes which were duplicated prior to the last speciation events and may occur in a wide range of taxa affecting early branches of the tree.

Deep paralogs have the strongest effect on the phylogenomic analyses but they are mostly uncovered by reference databases. Some ciliate studies use to identify paralogs only reference databases or programs (Chen *et al.* 2015, Feng *et al.* 2015, Sun *et al.* 2017), but most of them followed the whole filtering procedure including single-gene trees (Gao and Katz 2014, Gentekaki *et al.* 2017, Jiang *et al.* 2019, Lasek-Nesselquist and Johnson, 2019). Rarely, all single-gene alignments are provided in an open-access repository (Rotterová *et al.* 2020), which is the best option as other researchers have access to the data.

Another option to deal with paralogs could be the application of the software package PhyloFisher (Tice *et al.* 2021). PhyloFisher was designed to construct, perform quality control, and analyze large phylogenomic datasets. It includes a manually curated starting set of orthologs and their related paralogs from various eukaryotic taxa. A user can also construct its own starting database from alternative sets of orthologs and paralogs and process them by the PhyloFisher workflow. The manual ortholog inspection from single-protein trees is also more feasible by means of PhyloFisher. The individual trees can be visualized, and the clades or sequences suspected to be paralogs are automatically highlighted and can be easily collected/removed.

An alternative method to avoid the paralog problem might be using only the set of genes that have remained in a single copy (without duplications) since the last ciliate common ancestor. Those sets of genes are also called universal single-copy orthologs (USCOs) and are increasingly identifying and benchmarking (Creevey *et al.* 2011). The usage of USCOs as standardized nuclear markers for DNA taxonomy was already proposed for metazoans (Eberle *et al.* 2020). Yet, Tice *et al.* (2021) found several paralogs in the USCOs based-datasets.

Potential future use of mitochondrial genes in discrimination of ciliate species

The mitochondrial SSU-rDNA has been analysed in relatively few ciliate species, which makes it currently insufficient for routine ciliate identification as the databases are unpopulated by reference sequences. In phylogenetics, the mitochondrial SSU-rDNA is informative and useful as an alternative to the more standard nuclear DNA molecular markers (Dunthorn *et al.* 2011, 2012a, 2014a; Gao *et al.* 2016a; Wang *et al.* 2017, 2020). Phylogenetic analyses based on the mitochondrial SSU-rDNA sequences revealed potential cryptic species of *Chilodonella uncinate* (Katz *et al.* 2011) and *Trithigmostoma* sp. (Wang *et al.* 2020) that were not recognized based on more conservative nuclear SSU-rDNA. On the other hand, this marker revealed less consistent relationships compared to nuclear SSU-rDNA and COI (cytochrome c oxidase) gene in the case of ciliates from the subclass Scuticociliatia (Zhang *et al.* 2019) and is often generally unsuitable for resolving deep ciliate relationships (Wang *et al.* 2020). Mitochondrial SSU-rDNA has fewer copies per cell and displays a higher polymorphism than its nuclear counterpart (Wang *et al.* 2020). This low copy number might be an advantage for studies analysing environmental DNA, where a high copy number can cause bias in ecological or diversity interpretations. Most of the abovementioned studies also indicate that mitochondrial SSU-rDNA is variable enough to discriminate ciliate species. Given that the reference databases have become distinctly populated, it may allow species identification in future molecular biodiversity surveys (Wang *et al.* 2020).

The mitochondrial COI gene was used in early ciliate DNA barcoding studies, successfully distinguishing several *Paramecium* (Barth *et al.* 2006) and *Tetrahymena* (Lynn and Strüder-Kypke 2006, Lynn *et al.* 2018, Doerder 2019) species. Later studies showed the COI gene possess a high degree of genetic diversity at single cell, species, and population levels compared to the nuclear markers (Lynn and Strüder-Kypke 2006, Gentekaki and Lynn 2009, Strüder-Kypke and Lynn 2010, Zhao *et al.* 2016, Jung *et al.* 2018, Park *et al.* 2019, Zhang *et al.* 2019, Wang *et al.* 2021) but these studies were done only on oligohymenophorean and spirotri-

chean species. It is problematic to design COI primers universal for all ciliates as their mitochondrial DNA is highly variable and may contain various inserts (Gray *et al.* 2004, Park *et al.* 2019). Other problems are the potential presence of different haplotypes (Zhao *et al.* 2013) and absence of this gene in certain ciliate lineages and species (Lynn 2008).

Potential of sequencing mitochondrial genomes

The typical ciliate mitochondrial genome is organized into linear chromosomes with a length of several tens of kilobases (20–70 kb) and terminating with telomeres (Suyama and Miura 1968, Goddard and Cummings 1975, Morin and Cech 1988, Swart *et al.* 2012). The ciliate mitogenome exhibits a considerable variation in genome structure and GC content (Barth and Berendonk 2011), which is typical for microbial eukaryotes (Smith and Keeling 2015) compared to the relatively uniform and conserved metazoan mitochondrial genomes (Smith 2016). Besides the SSU-rRNA and CO1 genes, a typical aerobic ciliate mitogenome has a LSU-RNA gene, five to eight transfer RNA genes, multiple respiratory chain genes (nicotinamide adenine dinucleotide, cytochrome b, and cyclooxygenase-2), ATP (adenosine triphosphate) synthase complex, protein transport, and maturation genes (Gray *et al.* 2004). The assembled mitochondrial genomes have already been used for comparative and phylogenetic analyses (Gao *et al.* 2018, Li *et al.* 2018) and distinguishing of the morphologically similar species from the subclass Scuticociliatia (Huang *et al.* 2021).

We propose an easy and scalable methodology of sequencing the entire mitochondrial genome should be developed for biodiversity studies in ciliates. This method would be broadly applicable to most ciliates, but would, of course, not be applicable to groups that lack mitochondrial genomes (e.g., Lewis *et al.* 2020). Such whole-mitochondrial genome sequencing could be done by first performing targeted gene capture – enriching the targeted genomic region (Albert *et al.* 2007, Gnirke *et al.* 2009) – and the sequencing of just the mitochondrial genes. These whole-mitochondrial data would provide numerous single-copy or low-copy genes, and thus would avoid the problems associated with the extensive paralogy of nuclear protein coding loci and facilitate inferring phylogenetic species/relationships with various methods (Kapli *et al.* 2017, Luo *et al.* 2018).

CONCLUSION

Individually our proposed methods – morphological investigations using fluorescence staining, and observing phylogenetic species using mitochondrial genome sequencing – could provide new insights and thus a better understanding of ciliate biodiversity. But their power can be amplified by linking them in a single pipeline. Such a scale-able and high-throughput pipeline could involve a fluorescence cell sorting and imaging step, followed by a single-cell mitochondrial genome sequencing step.

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