INTERNATIONAL
JOURNAL OF **SYSTEMATIC**
AND EVOLUTIONARY
MICROBIOLOGY

MICROBIOLOGY
SOCIETY

Techniques and tools for species identification in ciliates: a review

Jeeva Susan Abraham,[1] S. Sripoorna,[1] Swati Maurya,[1] Seema Makhija,[1] Renu Gupta[2] and Ravi Toteja[1,*]

Abstract

Ciliates are highly divergent unicellular eukaryotic organisms with nuclear dualism and a highly specialized ciliary pattern. They inhabit all biotopes and play crucial roles in regulating microbial food webs as they prey on bacteria, protists and even on microscopic animals. Nevertheless, subtle morphological differences and tiny sizes hinder proper species identification for many ciliates. In the present review, an attempt has been made to elaborate the various approaches used by modern day ciliate taxonomists for species identification. The different approaches involved in taxonomic characterization of ciliates such as classical (using live-cell observations, staining techniques, etc.), molecular (involving various marker genes) and statistical (delimitation of cryptic species) methods have been reviewed. Ecological and behavioural aspects in species identification have also been discussed. In present-day taxonomy, it is important to use a 'total evidence' approach in identifying ciliates, relying on both classical and molecular information whenever possible. This integrative approach will help in the mergence of classical methods with modern-day tools for comprehensive species description in future.

INTRODUCTION

Ciliated protists (Ciliates) are a highly diverse clade of eukaryotic micro-organisms that are morphologically very complex and highly differentiated taxa among single-celled organisms [1]. They exhibit high species diversity, with over 4000 free-living ciliates described [2]. Ciliates have a number of distinctive structural and functional features, such as their extremely complex subcellular and organellar structures, structurally and functionally different macronuclear and micronuclear genomes, sexual reproduction by conjugation, whole genome duplications, patterns of cortical structure with semi-autonomous mechanisms of inheritance and morphogenesis, evidence of epigenetic mechanisms, broad array of ecological niches and lifestyles, and rapid evolutionary radiation at the species population level owing to their short generation times [3]. Their role is immense as they are part of the microbial loop, function as recyclers, remineralizers of organic material in terrestrial and aquatic systems, they prey upon the bacteria and smaller protists and maintain the ecosystem balance. These distinctive characteristics make them extremely useful models in studies of cell biology, molecular biology, genetics, ecology and

evolution [4, 5]. They have been used extensively as model systems for assessing heavy metal toxicity in freshwater ecosystems [6–9], to detect stress-induced morphological anomalies [10–12] and in the expression of genes such as hsp70, superoxide dismutase and metallothionein [13–15].

There are generally two major approaches used for identification of ciliates: the traditional method of live cell morphology, fixation, staining and morphometrics and, more recently, DNA-based methods. Nowadays, taxonomy is more focused on an integrative approach combining classical and molecular approaches. Ciliate taxonomy was traditionally done using microscopic techniques involving observation of live or fixed cells with details of the ciliature or silver-line system. With improvements in optical microscopy and the wider application of silver impregnation methods, detailed descriptions of ciliature, oral apparatus, nuclear apparatus and ultrastructure have been done and various new species have been described in previous years [16–19]. In addition, due to advances in molecular taxonomy techniques, phylogenetic studies have become increasingly important in the last three decades [20–22]. The application of powerful and ultra-rapid nucleic acid

sequencing techniques for phylogenetic studies have been utilized to a great extent in taxonomic studies.

The present review aims to discuss the different morphological and molecular methods involved in identification of ciliate species along with various statistical approaches to delimit cryptic species. Also, the role of ecological and behavioural aspects in the taxonomic characterization of ciliate species has been reviewed. Further, to have a complete species description, an integrative approach is suggested combining morphological and molecular methods and statistical tools as well as complementary features involving ecology and behaviour.

CILIATE TAXONOMY

Classification of the phylum Ciliophora

For the classification of phylum Ciliophora, two main classification systems are widely used. One is given by Corliss [19], which is based on morphological characters and the other by Lynn [4], which is inferred mainly from ultrastructural characters and small subunit (SSU) rRNA gene sequences, which are broadly consistent at class level but differ widely at order or family levels [23]. According to Lynn [4], there are about 300 families and 57 orders of ciliates. As per the recently updated classification given by Adl *et al.* [24], the phylum Ciliophora is divided into two subphyla namely, Postodesmatophora and Intramacronucleata. Postodesmatophora includes two classes Karyorelictea and Heterotrichea. Intramacronucelata have been divided into two groups: SAL and CONThreeP. SAL includes Spirotrichea and Lamellicorticata (i.e. Armophorea and Litostomatea). The SAL group also includes three other ciliates, the species *Mesodinium*, *Cariacothrix* and *Phacodinium*, but their phylogenetic positioning is still doubtful. The group CONThreeP includes Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Prostomatea and Plagiopylea (Fig. 1) [24–27].

I. Classical/traditional approaches: morphology-based species identification

(a) Microscopic observations

Live cell observations

To observe live ciliates under the stereoscopic or phase contrast microscope, cells are picked and dropped on a non-greasy clean slide. It is difficult to observe the detailed ciliature in fast-moving ciliates, therefore cells are immobilized or slowed down first by using Protoslo (Carolina Biological Supply Co)/methyl cellulose/polyoxresin WSR. All of these increase the viscosity of the water, thereby slowing the movement of the organism. However, this method often changes the shape of the cells or causes premortal alterations in various cell structures, hence, is not recommended. The preferable method is thus to select and pick cells using an appropriate micropipette and place them on a slide in a very small drop of fluid. Excess water/medium is removed and the slide is covered with a coverslip after applying Vaseline/petroleum jelly at the corners of the coverslip. The petroleum jelly corners are pressed with a mounted needle

until the ciliates become slightly squeezed between the slide and the coverslip [28]. Also, there are immobilization techniques by means of commercially obtained microcompressors or do-it-yourself methods [29]. Photomicrographs can be recorded with a camera mounted on the microscope only after the cells are properly immobilized. Ciliature can be studied to some extent (Fig. 2).

Scanning electron microscopy (SEM)

SEM allows a three-dimensional view of the ciliates and details can be documented which are difficult to reveal with other methods. The procedure involves fixation for 30 min in Parducz fixative containing four parts 2 % osmium tetroxide (OsO$_4$) and one part saturated mercuric chloride (HgCl$_2$). The cells are then washed in 0.05 M Na-cacodylate buffer (five times) [28] (Fig. 3a, b). The cells are transferred with a drop of buffer to the preparation chamber. The chamber is then dehydrated using alcohol series and dried in critical-point drying apparatus and coated with gold. Prepared cells are viewed at ×10 000 magnification.

(b) Staining procedures

Silver impregnation method

Many cytological techniques are useful for studying the morphology of protozoa, but the most widely used among them is the silver impregnation method. Silver impregnation is undertaken to reveal the ciliature and the silverline patterns (Fig. 3c–e and Fig. 4). Major techniques followed are silver carbonate staining [30], silver nitrate staining [31] and silver proteinate/protargol staining [32]. These impregnation protocols have been further modified and used according to different classes of ciliates [28, 32–36].

The wet silver nitrate method (Chatton and Lwoff staining) works well with different kinds of ciliates, especially with hymenostomes (e.g. *Tetrahymena*, *Paramecium*, *Cyclidium*), holophryids (e.g. *Holophrya*), most colpodids (e.g. *Colpoda*, *Bresslauides*) and some euplotids (e.g. *Euplotes*). Less convincing results are usually obtained with peritrichs (e.g. *Vorticella*), heterotrichs (e.g. *Spirostomum*, *Metopus*), oligotrichs (e.g. *Halteria*) and most hypotrichs (e.g. *Oxytricha*, *Urostyla*). Wet methods provide valuable information on the somatic and oral ciliature as well as the silverline networks (dorsal argyrome) which are, however, often rather faintly stained. The shape of the cells is usually well preserved, which is of advantage to the investigation (Fig. 3c–e). Modifications have been described by various authors [37–39].

The silver proteinate impregnating technique (protargol) serves to reveal the morphological key features of the ciliates such as the ciliature as well as the nuclear apparatus, extrusomes and various fibers (e.g. nematodesmata, basal body appendages etc). This is an impregnation technique, not a colouration technique whose impregnating agent is the silver proteinate which stains the kinetosomes or basal bodies of the cilia. It is also important to mention that protargol is no longer commercially available and needs to be synthesized in-house by the protocol suggested by Pan *et al.* [40] with improvements [41, 42]. The impregnation procedure involves fixation of ciliates in Bouin's fixative/Stieve's fluid/
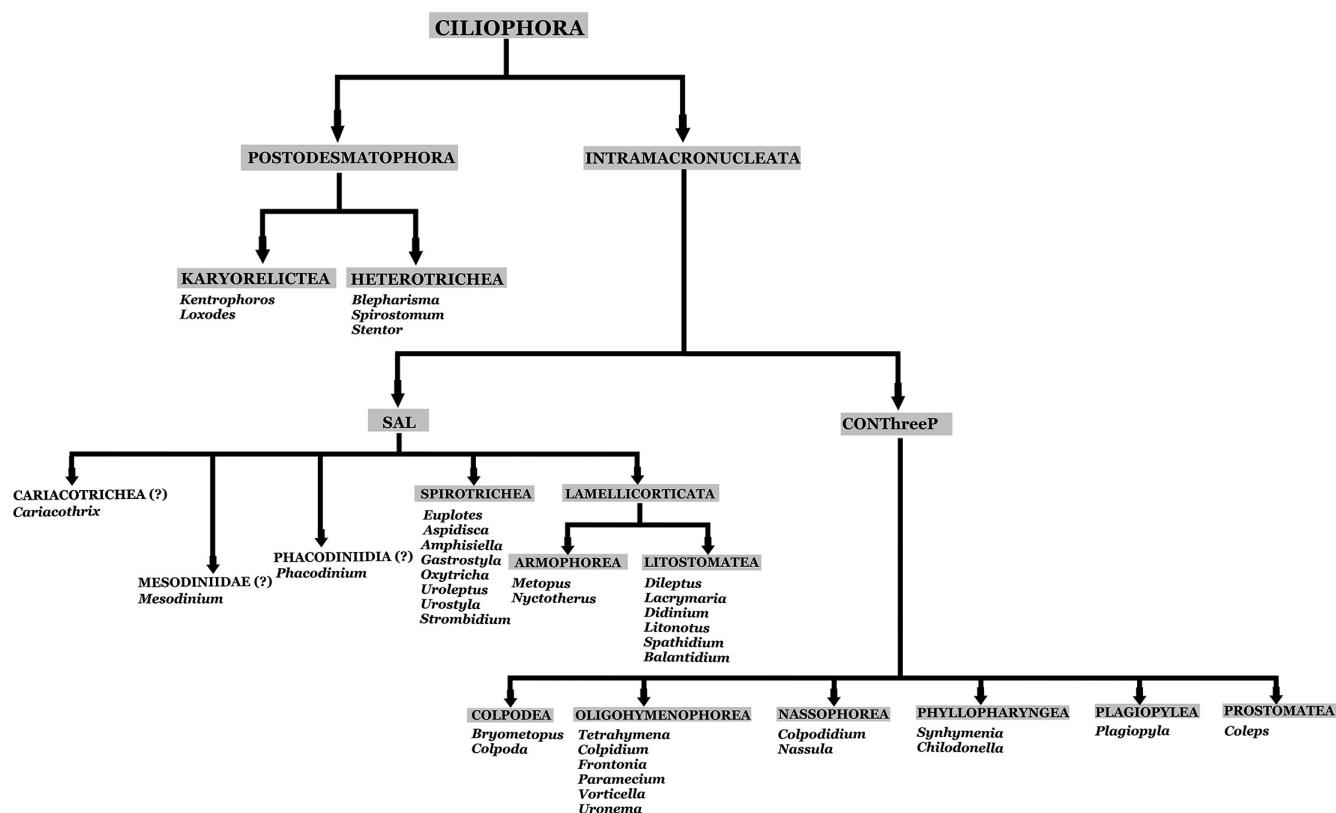
Fig. 1. Classification of the phylum Ciliophora (adapted from Adl *et al.* [24]). (?) in the figure indicates *insertae sedies* of *Cariacothrix*, *Mesodinium* and *Phacodinium* in the SAL group.

ethanol/formalin, etc. [28, 43], followed by enrobement in Meyer's albumin, coagulation of the albumin film by form-aldehyde–methanol solutions, bleaching in sodium hypo-chlorite (3 %), staining in protargol (0.8 %) for 30 min and lastly developing the stain and dehydration, clearing in xylene and mounting in DPX/Canada Balsam. To locate the macro- and micronuclear nodules or the cytoplasmic inclu-sions clearly, the pH of the protargol solution has to be adjusted by adding ammonium hydroxide (28–30 %) drop-wise [44]. The entire procedure is time consuming, but can be used to study the key morphological features of the cili-ates such as the pattern of the buccal ciliature, i.e. the undu-lating membranes and the adoral zone of membranelles, the pattern of the dorsal ciliature, i.e. the number and develop-mental pattern of the dorsal kineties, number and origin of marginal cirral rows, number of caudal and extra cirri pres-ent or absent, the pattern of the ventral ciliature, in particu-lar the number of the frontal-ventral-transverse (FVT) cirri and arrangement of frontoventral cirri (Fig. 4). These pat-terns are genus- and species-specific, as illustrated by Berger and Foissner [45] and Shao *et al.* [46].

FLUTAX staining of ciliates

This methodology has recently been proposed [47, 48] as a simple and easy alternative to silver staining methodologies to identify ciliates. Images obtained after FLUTAX staining

reveal the key characteristic features useful for identification of the ciliate species (Fig. 3f, g). The staining successfully shows the main ciliature patterns and location of structures important for the taxonomic identification of ciliates. FLUTAX (7-O-[N-(4-fluoresceincarbonyl)-L-alanyl] taxol) is a fluorescent derivative of taxol (Paclitaxel) that binds specifi-cally to microtubules and, since the main structures with taxonomic value in ciliates are microtubular, this method is valuable. It can also be used as vital staining since it does not always require previous fixation or permeabilization, although a permeabilization process might provide better results in some ciliates such as hypotrichs, which are nor-mally difficult to stain with silver impregnation due to their fragility [47].

For visualization of the microtubular system, derivatives of FLUTAX, i.e. FLUTAX 1 and 2 (defluorated variant of FLUTAX 1) are used. For FLUTAX 1 staining, single cells are incu-bated with 1 µM FLUTAX 1 for 10 min and placed in a drop of antifading agent which facilitates FLUTAX entry into the cells. Then, cells are mounted with a coverslip and observed immediately with an epifluorescence microscope [48]. For FLUTAX 2 staining, ciliates are permeabilized by using 0.5 % saponin in PHEM buffer [49] and are fixed in 2 % paraformaldehyde dissolved in PHEM buffer for 10–
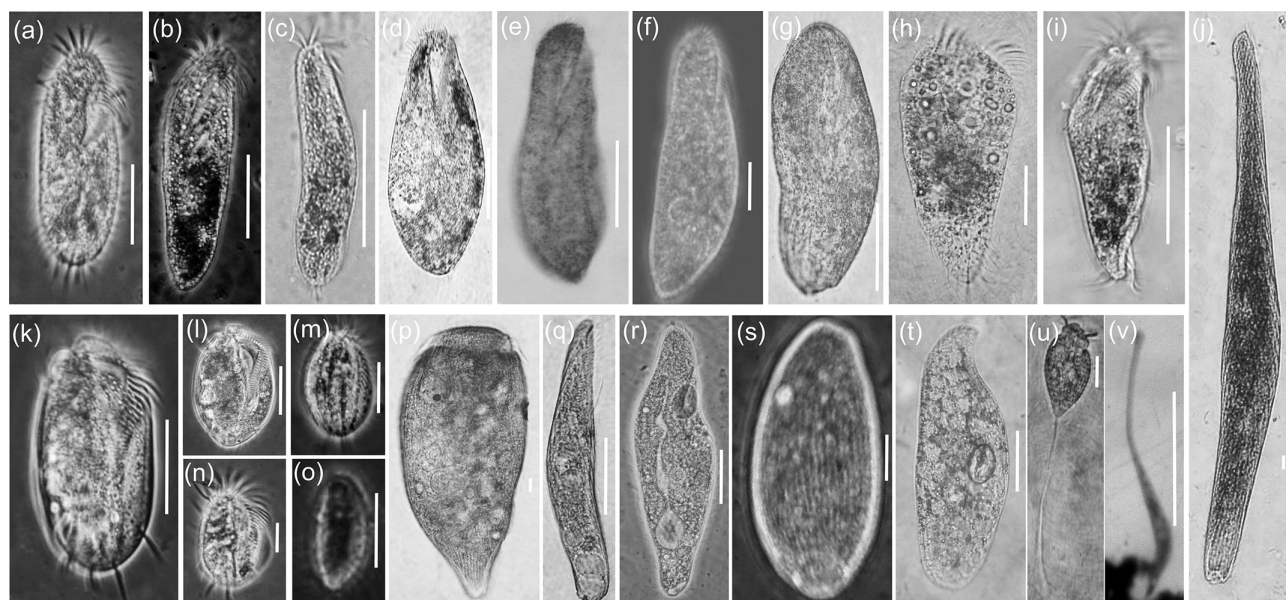
Fig. 2. Live cell images of selected fresh water ciliates isolated from Delhi, India. Species: (a) *Tetememena*; (b) *Aponotohymena*; (c) *Oxytricha granulifera*; (d) *Paraurostyla coronata*; (e) *Pseudokeronopsis*; (f) *Pseudourostyla*; (g) *Urostyla*; (h) *Laurentiella*; (i) *Gastrostyla*; (j) *Spirostomum*; (k) *Euplotes aediculatus*; (l) *Euplotes woodruffi*; (m) *Aspidisca*; (n) *Euplotes*; (o) *Cyclidium*; (p) *Stentor*; (q) *Blepharisma*; (r) *Paramecium*; (s) *Frontonia*; (t) *Loxodes*; (u) *Vorticella*; (v) *Lacrymaria*. Bars a–f, n, o, 20 µm; g–m, q–v, 50 µm; p, 100 µm.

15 s prior to incubation with FLUTAX 2 [47]. These preparations can be observed immediately or stored under cold, dark and wet conditions for a few days. Preparations are stable as long as they are not exposed to the wavelength exciting the fluorochrome. Once exposed, the fluorescence is quickly exhausted and the preparation loses its integrity;
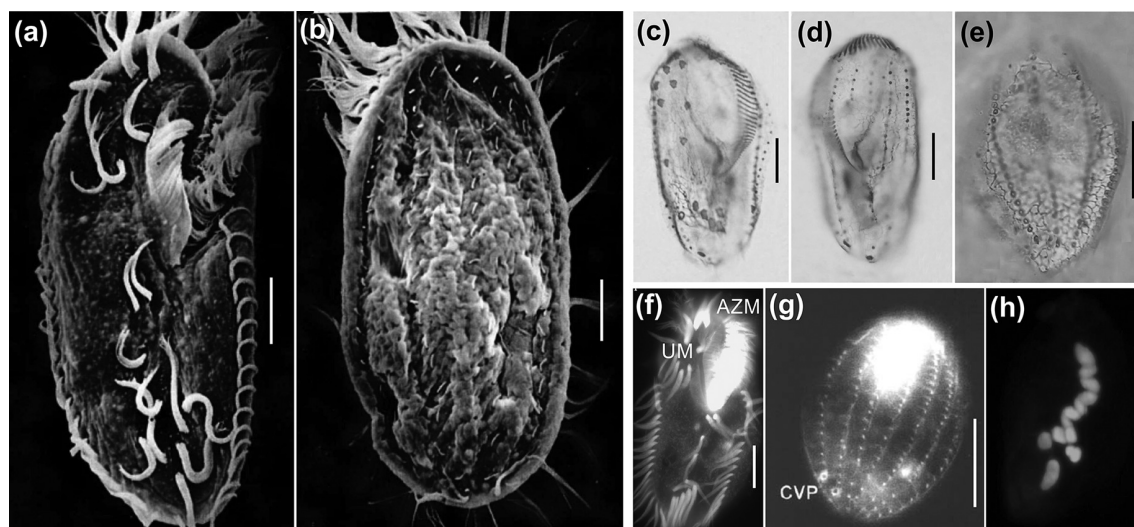
Fig. 3. (a-h) Photomicrographs depicting different techniques used for ciliate identification. (a–b) Scanning electron micrographs of (a) ventral surface and (b) dorsal surface of *Tetmemena*. (c–e) Wet silver nitrate staining of (c) ventral surface, (d) dorsal surface of *Euplotes aediculatus* and (e) dorsal surface of *Euplotes encysticus*. (f–g) FLUTAX staining revealing the ciliary structure of (f) *Sterkiella cavicola* (ventral view) and (g) *Tetrahymena thermophila* (dorsal view) [(f) and (g) adapted from Arregui *et al.* [47] and [48], respectively]. (h) Fluorescence *in situ* hybridization and 4,6–diamidino-2-phenylindole (DAPI) staining of *Condylostoma* stained with the Cy3-labelled probe Pspe210 and DAPI with 20 % formamide concentration [(h) adapted from Zhan *et al.* [53]]. AZM, adoral zone of membranelles; UM, undulating membrane; CVP, contractile vacuole pores. Bars, 20 µm.
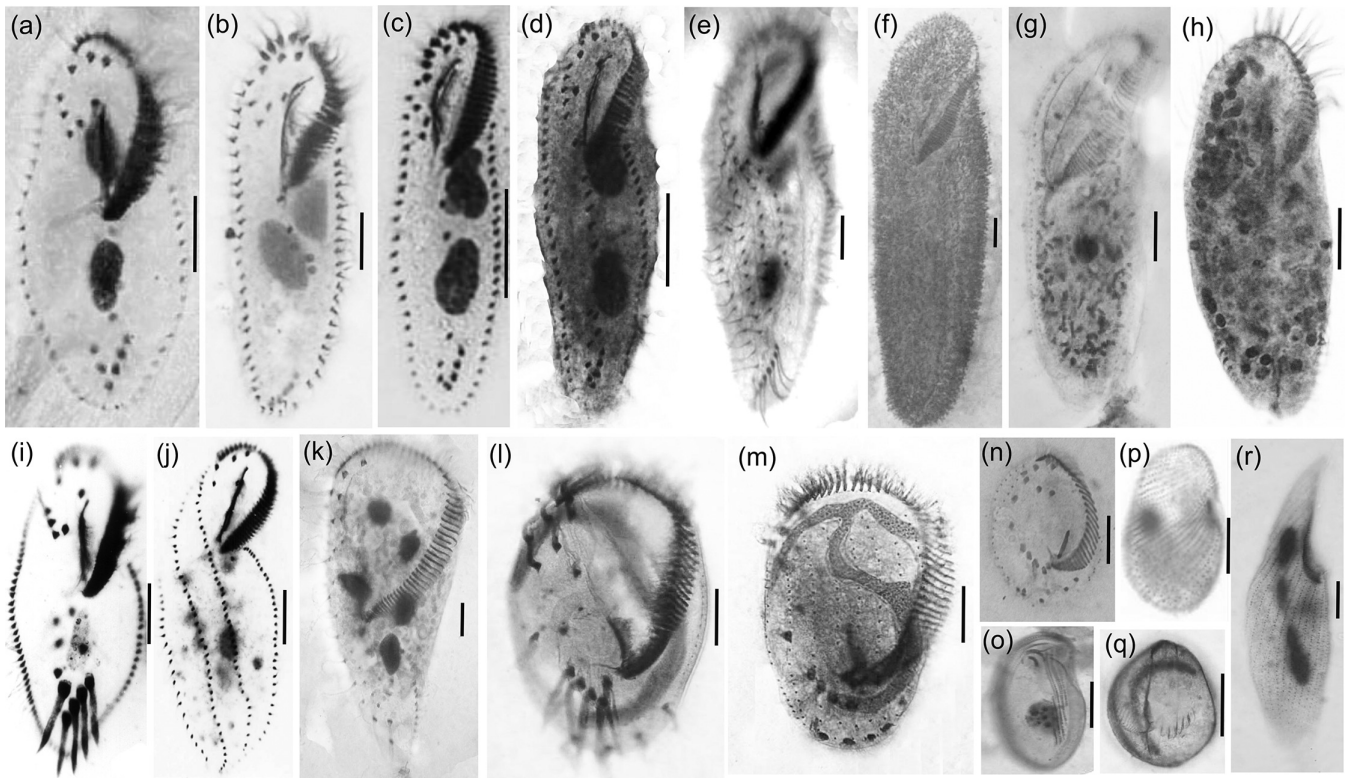
Fig. 4. Protargol-impregnated images showing ventral surface of selected ciliates. Species: (a) *Tetmemena*; (b) *Notohymena*; (c) *Oxytricha granulifera*; (d) *Gastrostyla*; (e) *Paraurostyla coronata*; (f) *Pseudourostyla cristata*; (g) *Urostyla*; (h) *Anteholosticha*; (i) *Histriculus histrio*; (j) *Strongylidium*; (k) *Laurentiella*; (l) *Euplotes aediculatus*; (m) *Euplotes woodruffi*; (n) *Euplotes*; (o) *Chilodonella*; (p) *Colpoda*; (q) *Aspidisca*; (r) *Blepharisma*. Bars, 20 µm.

hence, no permanent slides can be kept, which is one major drawback of this technique. Another drawback is that not all the subpellicular microtubules associated with the infraciliature are visualized. Apart from these disadvantages, efficiency, ease of use and rapidity of this method show that it is a good technique for ciliate species identification [47, 48].

Fluorescence *in situ* hybridization (FISH)
The FISH approach has the potential to allow easy and unequivocal identification of ciliates within complex samples by simple yes/no detection (getting a signal or not) [50]. Fluorescent probes have been used for a long time in the field of bacterial taxonomy [51], but this technique has seldom been used to identify ciliates without the need to refer to morphological characters. The new protocol provided by Fried *et al.* [52], is an adaptation of FISH combined with silver staining protocols to rapidly detect and identify ciliates. Another advantage of FISH is that, once a probe has been tested, no cultivation and sequencing is needed to identify cells in complex samples and sequence-based phylogenetic conclusions can be assigned to a morphotype *in situ* [53] (Fig. 3h). It can be used easily to monitor ciliates and other protists living in different

environments and the ecological function of particular ciliate species can be studied using grazing experiments (addition of stained food organisms) to the predator species [54]. The FISH technique implies the use of various fluorochrome-labelled oligonucleotide probes which hybridize to a target sequence within an intact cell, resulting in coloured signals that are detected with a fluorescence microscope [53]. This technique has been applied successfully in some ciliates e.g. *Glaucoma scintillans* [52], *Glaucomides bromelicola* [55] and *Pseudocohnilembus persalinus* [53].

Quantitative protargol stain (QPS)
QPS is a promising technique to bridge the gap between the taxonomic and quantitative methods. This technique is based on protargol impregnations combined with a filtration method for cell enumeration. The quality of the stain is reproducible and ranks equally with established protargol techniques used in ciliate taxonomy. The method was first developed by Montagnes and Lynn [33] and then later on odified by Skibbe [56]. Skibbe's method reduced the total time of preparation from 15 to 27 h to less than 4 h. In addition to the staining of basal body structures, cilia and the nuclear apparatus of the protozoan cells, the QPS also
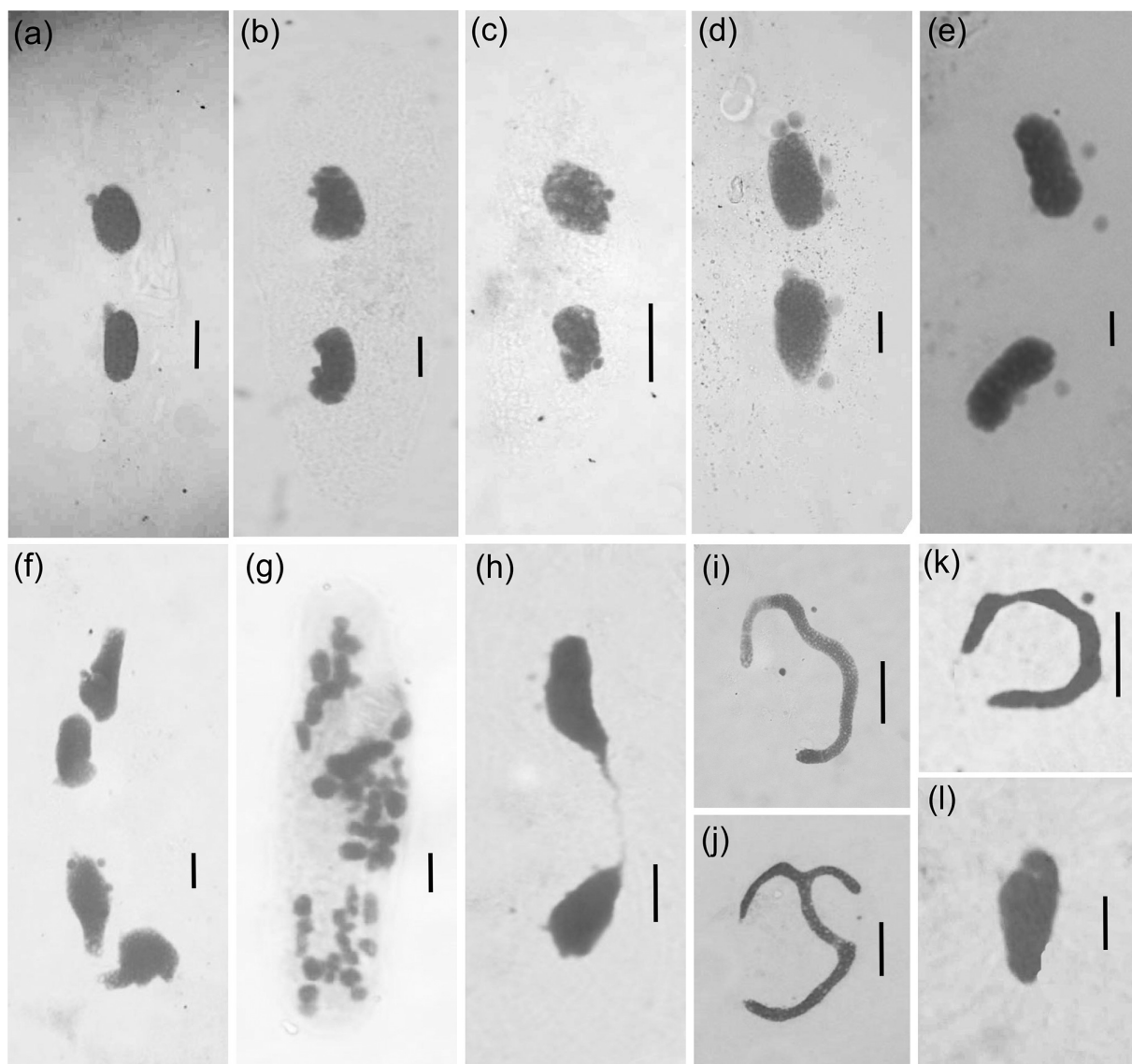
Fig. 5. Feulgen-stained nuclei of ciliates. Species: (a) *Notohymena*;, (b) *Gastrostyla*; (c) *Oxytricha granulifera*; (d) *Paraurostyla coronata*; (e) *Tetmemena*; (f) *Laurentiella*; (g) *Pseudourostyla cristata*; (h) *Blepharisma*; (i) *Euplotes aediculatus*; (j) *Euplotes woodruffi*; (k) *Euplotes*; (l) *Paramecium.* Bars, 10 µm.

provides information about the content of food vacuoles and presence of endosymbionts [56].

Feulgen, DAPI and methyl green pyronin staining for nuclei
Although the nucleus of the ciliates is stained well with the protargol, the Feulgen reaction is mainly used to reveal the nuclear apparatus very selectively as the protargol stains other cytoplasmic inclusions too sometimes and it becomes difficult to understand the nuclear morphology [28]. In this technique, DNA is stained specifically as the Schiff's reagent reacts with the aldehyde groups present in the deoxyribose molecules which are exposed by HCl hydrolysis. The staining intensity is also proportional to the DNA concentration [57]. This technique reveals the nuclear cytology in different

ciliate species, as illustrated in Fig. 5. The technique briefly involves fixation of cells for 15–20 min in Carnoy's fixative (four parts methanol and one part glacial acetic acid or three parts ethanol and one part glacial acetic acid), followed by adding one or two drops of fixed cells onto a clean glass slide with a dropper. After the cells are dried and fixed on slides, they are washed with distilled water. Further, acid hydrolysis is done by keeping the fixed slides in 1 N HCl for 7 min at 60 °C. Again, slides are washed and stained in Feulgen stain for 25–30 min, followed by differentiation in water and dehydration in alcohol series (30 %, 50 %, 70 % for 5 min each; 90 %, 100 % for 10 min each), clearing in xylene (10–15 min) and mounting in DPX/Canada Balsam [58].
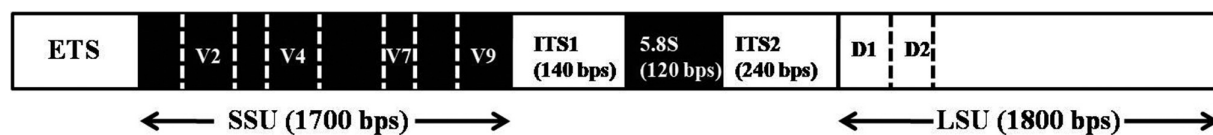
Fig. 6. General organization of the eukaryotic ribosomal genes showing hypervariable regions in the small and large subunit rRNA genes. ETS, external transcribed spacers; SSU, small subunit; ITS, internal transcribed spacer; LSU, large subunit.

DAPI (4′, 6-diamino-2-phenylindole dihydrochloride) is a fluorescent dye that binds to nuclear DNA. The fluorochrome is excited by UV light and can be observed with epifluorescence microscopy. The procedure involves fixing the cells with Lugol's solution [59]. Sodium thiosulphate is added to bleach the fixed sample and reduce darkness of the Lugol's fixed cells, which enhances the brightness of the fluorescence. Cells can also be fixed with either 8 % paraformaldehyde/0.2 % Triton-X 100 in a 1 : 5 ratio, or 20 % paraformaldehyde/50 % RNALAter/5 % Trizol in a 1 : 6 ratio for 15 min on microscopic slides and washed [60]. Further, 1 µg ml^{-1} DAPI solution is added to the sample and incubated for 3–4 min in the dark. This is then observed with epifluorescence microscopy using a UV filter (365 nm). DAPI staining is a rapid and reliable technique to reveal the nuclei in ciliates, but certain precautions need to be taken to effectively use it. For example, if higher concentrations of DAPI are used, it may stain the cytoplasm and the nucleus will not be distinguishable. Bleaching using sodium thiosulphate needs to be done in a controlled way otherwise the cells will become colourless and difficult to be traced. Also, DAPI-stained samples should be kept away from bright light, as DAPI is light sensitive and fades quickly, hence it not recommended for long-term preservation [53, 61, 62].

The methyl green pyronin method is a simple, quick and easy technique wherein nucleic acids such as DNA and RNA are differentially stained in green and red, respectively, and thus the nuclear morphology is revealed. But it also stains mucocysts in many ciliates (except those of tetrahymenids and many haptorids). The methyl green/pyronin mixture is prepared from 37 ml 0.5 % aqueous pyronin, 13 ml purified 0.5 % aqueous methyl green and 50 ml acetate buffer pH 4.8 (119 ml 0.2 M sodium acetate and 81 ml 0.2 N acetic acid) [63]. The technique involves mixing of one drop of fluid containing ciliates with one drop of methyl green pyronin and mixing the drops by gently swivelling the slide [28, 43]. The drawback of this technique is that it does not specifically stain DNA; therefore, there can be difficulties in observing the nucleus, especially the micronucleus since they are very small in size and there can be more than one in many ciliate species.

II. Molecule-based species identification

Molecular systematics is an emerging field of study in the area of ciliate systematics and often preferred over morphological data. The fact that many genes accumulate mutations at different rates and this difference is based on how much a gene can tolerate without losing its function is the basis for studying molecules in systematics. For example, histone molecules may become non-functional if some amino acids are replaced with different ones. On the other hand, the internal transcribed spacers (ITS) of rRNA can still fold properly even if many of its nucleotides are changed. Thus, ITS can accumulate mutations more rapidly than the histones. Therefore, such genes are used as molecular markers in studying ciliate systematics. In some cases, the morphological data becomes insufficient to distinguish two organisms at phylum, class, order, family, genus or species levels, thus analysis of the biomolecules is considered, which are large in number and can provide more accurate information to differentiate between two morphologically similar species. In other words, molecular information gives resolution below the species level [64]. Molecular data are easier to obtain and sometimes the only information available to trace the evolutionary history of a species. Each of the nucleotide variations should be considered as a character and is therefore an independent entity [65]. Thus, molecular identification has been proposed to serve as an alternative taxonomic approach for identifying the immense diversity of living organisms [66–68].

Small subunit (SSU) and large subunit (LSU) rRNA genes
The SSU rRNA gene was the first molecular marker to be employed [69]. Ribosomal RNA genes are one of the best molecules to be used till date for studying taxonomy because they are universal and are composed of highly conserved as well as variable regions [70]. In all eukaryotic organisms, the ribosome consists of SSUs having the 18S rRNA gene and the LSU with 5.8, 5 and 28S rRNA molecules (Fig. 6). rRNA genes are evolving more slowly than the protein-coding genes and are therefore particularly important in phylogenetic analysis. Numerous studies suggest the use of SSU rRNA gene sequences of ciliates to examine phylogenetic positions among them [71–73]. Currently, SSU rRNA gene sequences comprise the largest assemblage of ciliate data. In recent years, it has become clear that the SSU rRNA gene might be too conserved to uncover cryptic species of ciliates [74–76].

ITS sequences and 5.8S sequence data (ITS1–5.8S–ITS2)
Considerable effort has been dedicated to the search for alternative gene markers that will adequately reveal genetic diversity and provide better resolution especially at the tips of the ciliate tree. Additional molecular markers that are increasingly used for investigating phylogenetic

relationships among the ciliates include the internal transcribed sequences and 5.8S sequence data (ITS1-5.8S-ITS2) and tree reconstruction using them [77, 78]. The external transcribed spacer (ETS) and the internal transcribed spacers (ITS1 and ITS2) are the flanking regions of the SSU and the 5.8S rRNA which do not become part of the mature rRNA but their sequences and structures are required for the correct processing. ITS1 is located between SSU rRNA and 5.8S rRNA, and ITS2 is located between 5.8S rRNA and LSU rRNA [79] (Fig. 6). They accumulate a higher number of variations and are therefore suitable markers for phylogenetic studies [70]. Also, ITS1 is found to be more variable than the ITS2 gene, indicating that ITS1 is more suitable for species-level identification. Thus, SSU rRNA genes, LSU rRNA genes and ITS1-5.8S-ITS2 have been used extensively in phylogenetic analyses [64, 80].

Species identification by using phylogenetic analyses involves selection of a single or multiple marker genes preferably the most frequently used SSU rRNA, ITS1-5.8S-ITS2, LSU rRNA, mitochondrial COI (cytochrome oxidase I) gene [76] or others such as actin [81], α-tubulin [82], β-tubulin [81], elongation factor 1α [83] and histone 4 [84, 85]. PCR is performed for the particular marker gene selected with the help of specific primers (Table 1). The sequences obtained are used to reconstruct phylogenetic trees by using different software. Tree-building software that are commonly used are MEGA, PHYLIP, PAUP, PhyML, etc. The reconstructed trees are then viewed and analysed to understand the relationships among the species of interest [23, 86].

DNA barcoding
DNA barcoding is a technique that involves the use of short standardized DNA regions to identify species [87]. Many molecular marker genes such as hypervariable regions (V4, V9) of 18S rRNA gene, COI gene, ITS1-5.8S-ITS2 region and the D1–D2 region of the LSU rRNA have been suggested as barcoding genes [64] (Fig. 6). This technique involves the method of amplifying and sequencing these short stretches of DNA directly from the environment employing high throughput sequencing techniques such as 454 pyrosequencing and Ilumina/Solexa sequencing for estimating the complete diversity [88, 89]. It yields a million sequence reads in a single run, but results in much shorter sequences >500–700 bp at the most [90]. Because of this length limitation, these studies target short segments of DNA such as the hypervariable regions.

i. Hypervariable regions of the 18S rRNA gene
The entire region of 18S rRNA gene is around 1771 bp long (except in Litostomatea with 1635–1641 bp [40, 91], but there are specific stretches of DNA which are variable and are used in species identification. Prime candidates are the eight hypervariable regions V1–V5 and V7–V9. The V4 region is the longest of all regions: it comprises about 253.5 bp and correlates well with the entire sequence of the 18S gene with respect to the sequence information. The V2 region also correlates well, but is shorter with 161.8 bp on

an average. All other hypervariable regions are shorter than 100 bp and have lower correlation values [92]. The V9 region is also used immensely in high throughput sequence analyses of eukaryotic diversity [88, 93, 94] and has been suggested as a prime candidate especially for measuring protist lineage richness [94]. This is because V9 is of comparatively shorter length (75–150 bp) and has apparently lower sequencing error as compared to the longer V4 region [93]. It appears to yield less biased results across the broad taxonomic groups with general eukaryotic primers [89].

ii. Mitochondrial cytochrome c oxidase subunit I (COI) gene
The utility of mitochondrial cytochrome c oxidase subunit I (COI) gene in DNA barcoding studies was first introduced by Hebert and colleagues [87, 95]. The cytochrome c oxidase is a mitochondrial protein and is located in the inner mitochondrial membrane. COI is one of the most conserved mitochondrial protein-coding genes in animals and thus has a phylogenetic importance [96]. Additionally, mitochondrial genes have higher evolutionary rates than nuclear coded genes (e.g. SSU rRNA) and should be better suited to discriminate between closely related taxa [75, 97]. Even though it has been successfully used as a barcoding gene in many animal groups, its utility in protists has been studied to some extent in the recent years [98–100]. It was first successfully sequenced in ciliates species *Tetrahymena* and *Paramecium* (class Oligohymenophorea) [101, 102]. The COI gene is on average 2000–2200 nt long and includes an insert of about 300 nt long, which is used as a barcode to discriminate species [87]. The COI gene shows high divergence within ciliates and the insert region which is present in all classes is even more divergent. The resolution of COI at the intraspecific level is much greater than that of any nuclear genes and shows great potential to identify species on the basis of molecular data and to resolve the relationships of closely related ciliate taxa and uncover cryptic species. However, it is worthy to note that no universal primer sets of the COI gene are available to cover all groups of ciliate species [74, 103]. In the case of some ciliate groups, especially Litostomatea, Armophorea and some spitrotrich ciliate species, universal primer sets of the COI gene do not work [75]. In particular, ciliates like the armophoreans which inhabit anoxic ecosystems [4] and thereby lack functional mitochondria (e.g. functional COI gene) [103]. Recently, a new set of COI primers was designed to amplify this gene successfully in all spirotrichean ciliate species by using an *in silico* approach (Table 1). COI amplicons of 478 bp in around 69 populations of spirotrich ciliates were successfully obtained [103].

Secondary structures of hypervariable regions and ITS1 and ITS2 regions
Comparisons of secondary structures of hypervariable regions V2, V4, V7 and V9 of the SSU rRNA gene have been used and they show high degree of variability. The structures have been found to vary significantly within a genus and have been proved to be useful in species identification as well. In a study, Wang *et al.* [20] predicted the secondary structure of these four regions of the SSU rRNA

Table 1. List of primer pairs used for amplifying various marker genes for species identification

Target and primers	Sequences 5'–3'	Comments	References
Eukaryotic 18S rRNAs			
82F	GAADTCGYGAAYGGCTC	Universal	[153, 154]
EK-1520R	CYGCAGGTTCACCTAC		[153–155]
E528F	CGGTAATTCCAGCTCC	Universal	
1391R	GGGCGGTGTGTACAARGRG		
360FE	CGGAGARGGMGCMTGAGA	Universal	[153]
1492R	ACCTTGTTACGRCTT		
EU347F	AGGGTTCGATTCCGGAGA	Universal	[156]
EU929R	TTGGCAAATGCTTTCGC		
EukA	AACCTGGTTGATCCTGCCAGT	Universal	[16, 157]
EukB	TGATCCTTCTGCAGGTTCACCTAC		
Forward primer	CAACCTGGTTGATCCTGCCAGT	Universal	[76, 157]
Reverse primer	TTGGTCCGTGTTTCAAGACG		
Ciliophora 18S rRNAs			
Cil-f	TGGTAGTGTATTGGACWACCA	Cil-f amplifies 600–670bp of SSU with Cil-r I, II or III	[158]
Cil-r I	TCTGATCGTCTTTGATCCCTTA		
Cil-r II	TCTRATCGTCTTTGATCCCCTA		
Cil-r III	TCTGATTGTCTTTGATCCCCTA		
121F	CTGCGAATGGCTCATTAMAA	121F and 1147R amplify 1000 bp of 18S rRNAs	[154]
384F	YTBGATGGTAGTGTATTGGA	384F and 1147R amplify 750 bp of 18S rRNAs	
1147R	GACGGTATCTRATCGTCTTT		
Internal forward	CGGTAATTCCAGCTCCAATAG	For internal region of 18S rRNAs (710 bp amplicon size)	[16]
Internal reverse	AACTAAGAACGGCCATGCAC		
18S-6-CIL-V	AAYCTGGTTGATCCTGCCAG	Used in *Glaucoma* and *Tetrahymena* species	[52]
18S-1511-CIL-R	GATCCWTCTGCAGGTTCACCTAC		
mtF	TGTGCCAGCAGCCGCGGTAA	For mitochondrial SSU-rDNA (mtSSU-rDNA)	[129, 159]
mtR	CCCA(C)TACCA(G)GTACCTTGTGT		
mt400F	AAACTTAAAA(G)AAAT'TGGCGGGA	For mtSSU-rDNA in class Phyllopharyngea	[159]
mt900R	GAGGCGTGATGGGCGGGTGTGTGCA		
Eukaryotic 18S rRNAs (variable regions)			
TAReuk454FWD1	CCAGCA(G/C)C(C/T)GCGGTAATTCC	For V4 region	[89]
TAReukREV3	ACTTTCGTTCTTGAT(C /T)(A /G)A		
F-566	CAGCAGCCGCGGTAATTCC	For V4 to V5 region	[160]
R-1200	CCCGTGTTGAGTCAAATTAAGC		

Table 1. cont.

Target and primers	Sequences 5′–3′	Comments	References
Eukaryotic 18S rRNAs			
563f	GCCAGCAVCYGCGGTAAY	For *V4* to *V5* region	[161]
574f	CGGTAAYTCCAGCTCYAV		
616f	TTAAAAVGYTCGTAGTYG		
1132r	CCGTCAATTHCTTYAART		
897f	AGAGGTGRAATTCTHRGA	Amplifies V5 and V7 region with 1423 r	[161]
1132f	AYTTRAAGDAATTGACGG	Amplifies V7 with 1423r	
1266f	RGTGGTGCATGGCCGYTB	Amplifies V7 with 1423r	
1423r	GGGCATYWCDGACCTGTT		
Ciliophora 18S rRNA gene (variable regions)			
E06_757_LSU	TCTGGACTGCGTAGCCTA	For V9 region	162[]
E06_757_SSU	AACTTCCCGCAATAGCTAGG		
Eukaryotic ITS1-5.8S-ITS2 primers			
ITS1	TCCGTAGGTGAACCTGCGG	Universal	163[]
ITS4	TCCTCCGCTTATTGATATGC		
Ciliophora ITS1-5.8S-ITS2 primers			
5.8SF	GTAGGTGAACCTGCGGAAGGATCATTA	Used in spirotrich ciliates	[77]
5.8SR	TACTGATATGCTTAAGTTCAGCGG		
ITSF	CGTAACAAGGTTTCCGTAGG	Used in *Paramecium* species	[164]
ITSR	TCCTCCGCTTACTGATATGC		
Eukaryotic/Ciliophora 28S rRNA genes			
28S-1F	ACCCGCTGAACTTAAGCAT	28S-1F works with both 28S-4R and Euk34r	[162, 165]
28S-4R	TTCTGACTTAGAGGCGTTCAG		
Euk34r	GCATCGCCAGTTCTGCTTACC		
Eukaryotic/Ciliophora 28S rRNAs (variable regions)			
D1–D2 fw1	AGCGGAGGAAAAGAAACTA	Universal	[70, 85]
D1–D2 fw2	ACAAGTACCDTRAGGGAAAGTTG	Universal	
D1–D2 rev1	TACTAGAAGGTTCGATTAGTC	Designed for vertebrates	
D1–D2 rev2	ACGATCGATTTGCACGTCAG	Universal, Designed for invertebrates	
D1–D2 rev3	GCATAGTTCACCATCTTTCG	Designed primarily for vertebrates	
D1–D2 rev4	GTTAGACTYCTTGGTCCGTG	Universal	
1274f	GACCCGTCTTGAAACACGGA	Used in meiobenthic organisms, amplifies D3–D5 region with 706 or 689r primers and only D3 with 1275r primer	[166]
1275r	TCGGAAGGAACCAGCTACTA		

Table 1. cont.

Target and primers	Sequences 5'–3'	Comments	References
Eukaryotic 18S rRNAs			
1480f	TAGGGGCGAAAGACTCG	Used in meiobenthic organisms, amplifies D4–D5 region with 706 or 689r primers	
706r	CGCCAGTTCTGCTTACC		[76]
689r	ACACACTCCTTAGCGGA		
Ciliophora cox1 gene			
Forward primer	ATGTGAGTTGATTTTATAGAGACAGA	Used in Tetrahymenine ciliates	
Reverse primer	GGDATACCRTTCATTTT		
Cox-F	TCAGGTGCTGCACTAGC	Used in *Tetrahymena* species	[167–169]
Cox-R	TAAACTTCAGGGTGACCAAAAAATCA		
CauCoxF	TCAGGAGCTGCMTTAGCTCCYATG	Used in *Paramecium* species	[164]
CauCoxR	TARTATAGGATCMCCWCCATAAGC		
F388dT	TGTAAAACGACGGCCAGTGGWKCBAAAGATGTWGC	For 5 classes of ciliates: Oligohymenophorea, Nassophorea, Colpoda, Spirotrichea, Heterotrichea. Works with R1184dT primer	[75, 163]
F199dT-A	TGTAAAACGACGGCCAGTTCAGGWGCTGCAHTAGC	For *Tetrahymena* species, Hymenostomes. Works with R1184dT primer	[167]
F199dT-B	TGTAAAACGACGGCCAGTTCAGGWGCTGCMTTAGCHACYATG	For Scuticociliate, Peniculine species. Works with R1143dT primer	[164]
F298dT	TGTAAAACGACGGCCAGTGCNCAYGGTYTAATNATGGT	For class Heterotrichea. Works with R1184dT primer	[75]
R1184dT	CAGGAAACAGCTATGACTADACYTCAGGGTGACCRAAAAATCA	–	[168]
R1143dT	CAGGAAACAGCTATGACTARTATAGGATCMCCWCCATAAGC		[164]
CiCO1 Fv2	GWTGRGCKATGATYACACC	For class Spirotrichea, amplifies 478 bp of CO1 gene	[103]
CiCO1 Rv2	ACCATRTACATATGATGWCC		

D=A/G/T, Y=C/T, R=A/G, W=A/T, M=A/C, B=C/G/T, V=A/C/G, H=A/C/T, K=G/T, N=A/G/C/T.

gene of 45 urostylids. Structural comparison has shown that the V4 region is the most effective in revealing interspecific relationships, while the V9 region is suitable at family or higher level. The V2 region is also informative but was found unable to reflect phylogenetic relationships at family or lower level. The V7 region was found to be least informative. Therefore, secondary structure analysis had also provided clues about the phylogenetic relationships of problematic genera and provides better understanding of their phylogeny.

ITS1 and ITS2 genes have specific folding patterns and loop formations which can be utilized in phylogenetic analyses and understanding the species identity. Comparisons can be done at genus and species level using the primary sequence [104]. The rate of evolutionary changes in the ITS1 and ITS2 is also more than 100 times higher than that of 18S rRNA gene, making it one of the most popular markers in phylogenetic studies [80, 105]. Although both the ITS1 and ITS2 contain sufficient conserved regions and variable regions, ITS2 seems to contain more information potentially useful for comparisons at family, order and even higher levels.

The putative secondary structures of ITS gene can be predicted using the default settings of the mfold web server (http://unafold.rna.albany.edu/?q=mfold) [106, 107]. The structures can then be edited using RnaViz 2.0 software which is a user-friendly, portable windows-type program for producing secondary structure drawings of RNA molecules [108].

Regions of the LSU rRNA gene other than the hypervariable region of the SSU rRNA gene and certain divergent regions of the large subunit rRNA gene have also been explored to identify up to species level and to differentiate between cryptic species [109, 110]. LSU regions have been found to have advantages such as a faster evolutionary rate than SSU regions, less intra clonal and intra individual variablilty than ITS and considerably higher universality than COI [111–113]. Recently, two adjacent regions, D1–D2 have been studied; an amplified product of around 800–1300 bp can be obtained for a broad range of species with some specific PCR optimizations [70, 85, 111]. Studies using the LSU region have already been successfully done in the diatoms, dinoflagellates, tintinnid ciliates, *Paramecium* species and *Frontonia* species [64] and therefore it has proven to be a good candidate barcoding gene, but further exploration in different group of ciliates needs to be carried out in future.

III. Statistical analysis based species identification

Variability is a phenomenon of life and it can arise due to geographical isolation or by genetic factors such as genetic drift and other evolutionary processes. Thus, the problem of cryptic species arises and thereby difficulty in revealing the hidden diversity of ciliates. Numerous strains need to be examined, preferably from widespread locations, to be confident that any significant differences observed are not simply due to local genetic variations. Reproductive isolation among species needs to be studied before assigning two species as different, but it is practically difficult especially when two species to be compared are located in geographically different locations and belong to different habitats. Hence, quantification and statistical analysis of the morphological descriptions are required in order to avoid bias [114, 115].

i. Multivariate morphometric analyses

The extent of morphological variation within and among populations need to be studied using multivariate techniques which is rarely used in ciliate taxonomy [115]. Advanced techniques for morphometric analysis provide more useful and powerful tools in identifying the differences between ciliate populations, detecting differences among groups, and to differentiate between same species. Multivariate morphometrics is a formidable tool for determination of patterns of variation at species level for ciliates. Morphometric methods have been developed and applied to discriminate various ciliate species. The use of multivariate techniques such as principal components and discriminant analyses to quantify morphometric variables are also receiving increased attention in ciliate taxonomy. Multivariate morphometrics comprises a wide spectrum of methodological approaches. The choice of a particular method or approach depends on the data being used, hypotheses to be tested and questions being asked.

Analysis of variations in quantitative measures of patterns of cirral placement on the ventral surface can be used to study the processes of population differentiation and can be interpreted to resolve taxonomic problems. Similarly, the stability of morphological structural patterns can be ascertained using such analyses [116]. *In vivo* as well as protargol-impregnated specimens are utilized for such analyses that provide a wealth of information required to delimit morphospecies, taking into account for variability. Recently, a multivariate approach was used to investigate the qualitative and quantitative morphometrical variation of five African *Semispathidium* species [115] and of six new Australian species of the genus *Metopus* [117]. Various methods like cluster analyses (CA) using the computer program SYNTAX 2000 [118], principal component analysis (PCA) and canonical discriminant analysis (CDA) were conducted using SAS software [119] to find out variations in the morphometric features such as number of ciliary rows, the length:width ratio of macronucleus, etc. Similarly, Irwin *et al.* [120] studied the diversification of mobilid Peritrichs and applied linear discriminant analysis (LDA) to assess the variation between two populations of *Trichodina domerguei*. Foissner and Schubert [121] applied discriminant analysis to separate two species of *Colpoda* (*Colpoda aspera* from *Colpoda elliotti*). Ginoris *et al.* [122] showed that discriminant analysis together with neural networks is a potentially alternative tools to identify taxa by computer analysis of their digital images.

ii. Bayesian delimitation approaches

It has been known that different genes or genomic regions may have different evolutionary histories (gene trees), due to several biological processes including the coalescent process in ancestral species, gene duplication and horizontal gene transfer [123–125]. Use of morphological data alone may thus underestimate the number of species and, in particular, may fail to identify cryptic species. Most of the sequence data for the closely related species or recently diverged species, for which species delimitation may be most problematic, will provide poorly resolved gene trees. Moreover, branch lengths on gene trees are important since they provide information about the ancestral population. These problems can be overcome by a Bayesian method for calculating the posterior probabilities of potential species delimitations using multilocus sequence data. This method provides support for different species delimitations using recently developed theoretical models that combine species phylogenies and gene genealogies via ancestral coalescent processes [126]. The method requires multiple sequences from multiple genes/loci of closely related species to statistically compare the genetic distances among them and assign posterior probability values. A unique feature of the method is that biologists can incorporate information on plausible species membership from morphology, paleontology and other sources by specifying different priors in the Bayesian model.

IV. Ancillary approaches to species identification

Ecology

Ciliate morphology is closely related to the function of the organism in nature; hence it is necessary to understand the role of ciliates in its particular environment, which is again related to the behaviour of the ciliate. In other words, to identify a species to its completeness it is important to have a proper knowledge about its surroundings. It has been known that many ciliate species co-occur in homogenous environments, indicating a wide range of their ecological niches. The ecological niche of a ciliate community comprises the ciliate species present in any given area along with the other abiotic and biotic factors interacting with the ciliate community inhabiting that area. The originality of a species and its functional distinctiveness are important for quantifying how a given species contributes to the functional diversity of a community and to ecosystem functioning [127]. In-depth studies involve the application of high-resolution phylogenetic, genomic and the most recent 'omic' analyses (metagenomics, metatranscriptomics, metaproteomics and communities' metabolomics) [128]. Application of the 'omic' techniques to the environmental samples will not only result in documentation of the rare/hidden species [129] but also their functional ecological roles. If a rare species is isolated from a rare habitat, how different is its genetic makeup from a similar species isolated from a common habitat is a question yet to be answered. For example, genomic comparison and gene expression analysis studies revealed that *Euplotes focardii* living in cold environments such as Antarctica and the

Arctic has lost the heat shock response modulated by the *Hsp70* gene with respect to mesophilic species of *Euplotes*, most likely due to constant cold environments in the Antarctic Ocean [130]. Similarly, comparative RNAseq analysis of *Tetrahymena thermophila* exposed to different concentrations of pesticides revealed how toxins might affect expression of conservative ribosomal proteins [131]. These modifications are excellent example of phenotypic plasticity in adaptation to physical parameters (i.e. low temperatures, concentration of pesticides, etc.), suggesting the role of gene–environment interactions. Therefore, physical parameters such as temperature, salinity and pH of the water, sediment or soil from which samples are taken should be valued and recorded [132].

Behaviour

Behaviour of ciliates is a complex phenomenon which not only involves cellular motility but other vitally important activities such as feeding, reproduction, sexual activity, avoidance of danger and search for safety, colonization of new habitats, etc. [133]. Such behaviour is formed out of the adaptive tricks that were used by various ciliate species in the past in order to overcome biological and environmental challenges. Thus, ciliate behaviour, in reality, is a response of the organism to its constantly changing environment. For example, capability of encysting to avoid extreme conditions of temperature or dryness or producing carnivorous giants [134] to overcome periods of severe starvation [135] are part of behavioural attributes with respect to the environment. Patterns of crawling or swimming, jumping and escape behaviour, feeding, contractility, formation of stalks, temporary attachment, alternation of rests and movement, are some important behavioural parameters which can be recorded. Soleymani *et al.* [136] studied usefulness of advanced movement features and morphological data in classifying different species of ciliates. This was done by using automated video analysis which recorded movement (e.g. ciliate trajectory, speed, linearity) and morphology (e.g. cell size, cell shape) in a number of individuals in order to classify ciliate species. It is also important to note that behaviour is dependent on their present environment (food type and abundance, temperature, pH, substrate type, light, etc.) and convergent behaviours are conserved across taxa and they are similar at least in congeners [132]. Thus, it can only be regarded as a complementary feature and not as a method for ciliate species identification.

Ricci devised original approaches and techniques to investigate the motile behaviour of ciliates. Central to his studies was the concept of the ethogram and of the ways in which it varies in different ciliates and under different conditions. Ethograms help us to find the relationships between the size and behaviour of ciliates. Study on locomotory behaviours of two *Uronychia* species (*Uronychia transfuga* and *Uronychia setigera*) indicated that the velocity and gripping behaviour in the locomotion varied in both the species depending on their sizes [137]. In a recent study, a multivariate approach, using 35 quantitative, 12 derived ratios and 3

qualitative variables documented that interspecies differences are bigger than intraspecies ones [138]. Consequently, it may be concluded that each species has a distinct type of locomotory behaviour. Detailed ethograms are, however, available for only a few ciliates [139–142]. A comparative study on locomotory behaviour of two predatory ciliates, *Coleps* and *Spathidium*, suggest that their locomotory behaviours are different where *Coleps* show similarity to that of *Tetrahymena* since they fall in the same cluster CONThreeP and *Spathidium* with *Litonotus* which falls in the same class Litostomatea. This study supports that locomotory behaviour reflects evolutionary history rather than cell geometry and/or predatory lifestyle [138].

V. Integrative approach for species identification

The present-day taxonomic approach is to integrate different aspects of biology into studying one concept [143]. The integrative approach aims to take full advantages of the advances in technology by combining them with traditional approaches [3, 144–146]. In simple words, integrative taxonomy is a mix of morphological and molecular methods for the identification of organisms [147]. It also provides a means of documentation of taxonomic descriptions that will provide the sort of 'portal' to other sources of information (e.g. genetic and ecological) that can contribute to the cross-disciplinary research that can link the events and draw inference on how the present-day biodiversity must have been shaped from that past events [132, 148] and maybe it can predict the future events that is yet to happen.

Some successful models for studying integrative taxonomy of ciliates have already been presented in oligotrich species *Novistrombidium testaceum* and *Strombidium inclinatum* where behavioural data, morphological data and sequences of the SSU rRNA gene are used for species redescription [149]. In another study, *Frontonia* species have been delineated by combining live observations, silver staining methods and genetic information from the cox1 subunit gene, nuclear ITS genes, hypervariable region D2 of the LSU rRNA gene and V2 and V4 hypervariable regions of the SSU rRNA gene [64]. *Spirostomum* species were also studied recently using multiple molecular markers (ITS1-5.8S-ITS2 region), the secondary structure of the ITS2 region and 18 and 28S (D1–D2) sequences [80]. The integrative approach has been used to understand the phylogeny of spathidiids [150], the positioning of the problematic taxon *Protocruzia* [21], the diversification of peritrich ciliates [151] and diversification of parasitoid apostomes, the pseudocollidae [152].

Gap in research and future perspectives

Since the microbial eukaryotic communities highly influence the health of the freshwater and soil ecosystems, there is a fundamental need to document the taxonomic composition of freshwater and soil ciliate diversity in unexplored regions or habitats using systematic biodiversity surveys. Attempts should be made to characterize unknown species of unexplored areas by using an integrative approach combining morphology and molecules. Thus, biodiversity research is the foundation of all kinds of higher and applied research. Comprehensive and time-to-time diversity estimates of different habitats and a biogeographic map for relatively common species need to be generated. This information is critical to manage and conserve the functional properties of the ecosystems for the long term, particularly in areas vulnerable to human activities. Considering the importance of ciliate taxonomy/biodiversity studies, funds need to be raised solely for studying this research area and more training opportunities need to be provided for young researchers to gain expertise in this field.

References
1. Chen X, Lu X, Luo X, Jiang J, Shao C *et al.* The diverse morpho-genetic patterns in spirotrichs and philasterids: researches based on five-year-projects supported by IRCN-BC and NSFC. *Eur J Protistol* 2017;61:439–452.
2. Gao F, Huang J, Zhao Y, Li L, Liu W *et al.* Systematic studies on ciliates (Alveolata, Ciliophora) in China: progress and achievements based on molecular information. *Eur J Protistol* 2017;61: 409–423.
3. Clamp JC, Lynn DH. Investigating the biodiversity of ciliates in the 'Age of Integration'. *Eur J Protistol* 2017;61:314–322.
4. Lynn DH. *The Ciliated Protozoa: characterization, Classification and Guide to the Literature*, 3rd ed. Netherlands: Springer-Verlag; 2008.
5. Hausmann K, Bradbury P. *Ciliates: Cells as Organisms.* Germany, Stuttgart: Gustav Fischer Verlag; 1996.
6. Somasundaram S, Abraham JS, Maurya S, Makhija S, Gupta R *et al.* Cellular and molecular basis of heavy metal-induced stress in ciliates. *Curr Sci* 2018;114:1858–1865.
7. Kim BM, Rhee JS, Choi IY, Lee YM. Transcriptional profiling of antioxidant defense system and heat shock protein (*Hsp*) families in the cadmium- and copper-exposed marine ciliate *Euplotes* crassu. *Genes Genomics* 2018;40:85–98.
8. Abraham JS, Sripoorna S, Choudhary A, Toteja R, Gupta R *et al.* Assessment of heavy metal toxicity in four species of freshwater ciliates (Spirotrichea:Ciliophora) from Delhi, India. *Curr Sci* 2017;113:2141–2150.
9. Amaro F, Turkewitz AP, Martín-González A, Gutiérrez JC. Whole-cell biosensors for detection of heavy metal ions in environmental samples based on metallothionein promoters from *Tetrahymena thermophila. Microb Biotechnol* 2011;4:513–522.
10. Makhija S, Gupta R, Toteja R. Lithium-induced developmental anomalies in the spirotrich ciliate *Stylonychia* lemnae (Ciliophora, Hypotrichida). *Eur J Protistol* 2015;51:290–298.

11. Makhija S, Gupta R, Toteja R, Abraham JS, Sripoorna S. Cadmium induced ultrastructural changes in the ciliate, *Stylonychia mytilus* (Ciliophora, Hypotrichida). *J Cell Tissue Res* 2015b;15:1–7.

12. Machwe S, Arora S, Gupta R, Sapra GR. Cisplatin induces modifications in the development of cell surface patterns of ciliates. *Cell Biol Int* 2001;25:1131–1138.

13. Toteja R, Makhija S, Sripoorna S, Abraham JS, Gupta R. Influence of copper and cadmium toxicity on antioxidant enzyme activity in freshwater ciliates. *Indian J Exp Biol* 2017;55:694–701.

14. Gutiérrez JC, Amaro F, Díaz S, de Francisco P, Cubas LL *et al.* Ciliate metallothioneins: unique microbial eukaryotic heavy-metal-binder molecules. *J Biol Inorg Chem* 2011;16:1025–1034.

15. La Terza A, Miceli C, Luporini P. The gene for the heat-shock protein 70 of *Euplotes focardii*, an Antarctic psychrophilic ciliate. *Antarct Sci* 1999;16:23–28.

16. Gupta R, Abraham JS, Sripoorna S, Toteja R, Makhija S *et al.* Taxonomic and morphogenetic description of the freshwater ciliate *Aponotohymena isoaustralis* n. sp. (Ciliophora; Oxytrichidae) isolated from Sanjay Lake, Delhi, India. *Acta Protozool* 2017;56:93–107.

17. Foissner W. Terrestrial and semiterrestrial ciliates (Protozoa, Ciliophora) from Venezuela and Galápagos. *Denisia* 2016;35:1–912.

18. Gupta R, Kamra K, Sapra GR. Morphology and cell division of the oxytrichids *Architricha indica* nov. gen., nov. sp., and *Histriculus histrio* (Müller, 1773), Corliss, 1960 (Ciliophora, Hypotrichida). *Eur J Protistol* 2006;42:29–48.

19. Corliss JO. *The Ciliated Protozoa: Characterization, Classification and Guide to the Literature*, 2nd ed. USA, New York: Pergamon press; 1979.

20. Wang P, Gao F, Huang J, Strüder-Kypke M, Yi Z. A case study to estimate the applicability of secondary structures of SSU-rRNA gene in taxonomy and phylogenetic analyses of ciliates. *Zool Scr* 2015;44:574–585.

21. Gentekaki E, Kolisko M, Boscaro V, Bright KJ, Dini F *et al.* Large-scale phylogenomic analysis reveals the phylogenetic position of the problematic taxon *Protocruzia* and unravels the deep phylogenetic affinities of the ciliate lineages. *Mol Phylogenet Evol* 2014;78:36–42.

22. Zhang Q, Yi Z, Fan X, Warren A, Gong J *et al.* Further insights into the phylogeny of two ciliate classes Nassophorea and Prostomatea (Protista, Ciliophora). *Mol Phylogenet Evol* 2014;70:162–170.

23. Gao F, Warren A, Zhang Q, Gong J, Miao M *et al.* The all-data-based evolutionary hypothesis of ciliated protists with a revised classification of the phylum Ciliophora (Eukaryota, Alveolata). *Sci Rep* 2016;6:1–14.

24. Adl SM, Bass D, Lane CE, Lukes J, Schoch CL *et al.* Classification of eukaryotes: revisions to the classification, nomenclature and diversity of eukaryotes. *J Eukaryot Microbiol* 2018 https://doi.org/.

25. Maurer-Alcalá XX, Yan Y, Pilling OA, Knight R, Katz LA. Twisted tales: insights into genome diversity of ciliates using single-cell 'omics. *Genome Biol Evol* 2018;10:1927–1938.

26. Lynn DH, Kolisko M, Bourland W. Phylogenomic analysis of *Nassula variabilis* n. sp., *Furgasonia blochmanni*, and *Pseudomicrothorax dubius* Confirms a Nassophorean Clade. *Protist* 2018;169:180–189.

27. Chen X, Wang Y, Sheng Y, Warren A, Gao S. GPSit: An automated method for evolutionary analysis of nonculturable ciliated microeukaryotes. *Mol Ecol Resour* 2018;18:700–713.

28. Foissner W. An update of 'basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa'. *Int J Syst Evol Microbiol* 2014;64:271–292.

29. Skovorodkin I. A device for immobilizing biological objects in the light microscope studies. *Tsitilogia* 1990;32:301–302. (in Russian with English summary).

30. Fernandez-Galiano D. Siver impregnation of ciliated protozoa: procedure yielding good results with the pyridinated siver carbonate method. *Trans Am Microsc Soc* 1976;95:557–560.

31. Chatton E, Lwoff A. Imprégnation, par diffusion argentique, de l'infraciliature des ciliés marins et d'eau douce, après fixation cytologique et sans desiccation. *CR Seances Soc Biol* 1930;104:834–836.

32. Wilbert N. Eine verbesserte Technik der Protargolimpänation für Ciliaten. *Mikrokosmos* 1975;64:171–179.

33. Montagnes DJS, Lynn DH. A quantitative protargol stain (QPS) for ciliates: method description and test of its quantitative nature. *Mar Microb Food Webs* 1987;2:83–93.

34. Wicklow BJ, Hill BF. A short procedure for protargol staining. In: Lee JJ and Soldo AT (editors). *Protocols in Protozoology*. Kansas, USA: Allen Press; 1992. pp. C-5.1–-5.5.

35. Foissner W. Protargol methods. In: Lee JJ and Soldo AT (editors). *Protocols in Protozoology*. Kansas,USA: Allen Press; 1992. pp. C-6.1–-6.0.

36. Lynn DH, Staining P. Protargol staining. In: Lee JJ and Soldo AT (editors). *Protocols in Protozoology*. Kansas, USA: Allen Press; 1992. pp. C-4.1–C-4.8.

37. Chatton E. Sur une méthode rapide d'impregnation a l'argent réduit par l'hydroquinone. *C R Seanc Soc Biol* 1940;134:229–232.

38. Frankel J, Heckmann K. A simplified chatton-lwoff silver impregnation procedure for use in experimental studies with ciliates. *Trans Am Microsc Soc* 1968;87:317–321.

39. Roberts DM, Causton H. Silver nitrate impregnation of ciliated protozoa. *Archiv für Protistenkunde* 1988;135:299–318.

40. Pan X, Bourland WA, Song W. Protargol synthesis: an in-house protocol. *J Eukaryot Microbiol* 2013;60:609–614.

41. Ji D, Wang Y. An optimized protocol of protargol staining for ciliated protozoa. *J Eukaryot Microbiol* 2018;65:705–708.

42. Kurilov AV. Improvement of silver impregnation technique using *in situ* synthesized Protargol. *Acta Protozool* 2017;56:109–118.

43. Foissner W. Basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa. *Eur J Protistol* 1991;27:313–330.

44. Kim JH, Jung J-H. Cytological staining of protozoa: a case study on the impregnation of hypotrichs (Ciliophora: spirotrichea) using laboratory-synthesized protargol. *Animal Cells Syst* 2017;21:412–418.

45. Berger H, Foissner W. Cladistic relationships and generic characterization of oxytrichid hypotrichs (protozoa, ciliophora). *Archiv für Protistenkunde* 1997;148:125–155.

46. Shao C, Lu X, Ma H. A general overview of the typical 18 frontal-ventral-transverse cirri oxytrichidae *s. l.* genera (Ciliophora, Hypotrichia). *J Ocean Univ China* 2015;14:522–532.

47. Arregui L, Muñoz-Fontela C, Guinea A, Serrano S. FLUTAX facilitates visualization of the ciliature of oxytrichid hypotrichs. *Eur J Protistol* 2003;39:169–172.

48. Arregui L, Muñoz-Fontela C, Serrano S, Barasoain I, Guinea A. Direct visualization of the microtubular cytoskeleton of ciliated protozoa with a fluorescent taxoid. *J Eukaryot Microbiol* 2002;49:312–318.

49. Schliwa M, van Blerkom J. Structural interaction of cytoskeletal components. *J Cell Biol* 1981;90:222–235.

50. Petroni G, Rosati G, Vannini C, Modeo L, Dini F *et al. In situ* identification by fluorescently labeled oligonucleotide probes of morphologically similar, closely related ciliate species. *Microb Ecol* 2003;45:156–162.

51. Amann R, Ludwig W. Typing *in situ* with probes. In: Priest FG, Ramos-Cormenzana A and Tindall BJ (editors). *Bacterial Diversity and Systematics*. Boston, MA: Springer; 1994. pp. 115–135.

52. Fried J, Ludwig W, Psenner R, Schleifer KH. Improvement of ciliate identification and quantification: a new protocol for

fluorescence in situ hybridization (FISH) in combination with silver stain techniques. *Syst Appl Microbiol* 2002;25:555–571.

53. **Zhan Z, Stoeck T, Dunthorn M, Xu K.** Identification of the pathogenic ciliate *Pseudocohnilembus persalinus* (Oligohymenophorea: Scuticociliatia) by fluorescence *in situ* hybridization. *Eur J Protistol* 2014;50:16–24.

54. **Gunderson JH, Goss SH.** Fluorescently-labeled oligonucleotide probes can be used to identify protistan food vacuole contents. *J Eukaryot Microbiol* 1997;44:300–304.

55. **Fried J, Foissner W.** Differentiation of two very similar glaucomid ciliate morphospecies (Ciliophora, Tetrahymenida) by fluorescence *in* situ hybridization with 18S rRNA targeted oligonucleotide probes. *J Eukaryot Microbiol* 2007;54:381–387.

56. **Skibbe O.** An improved quantitative protargol stain for ciliates and other planktonic protists. *Arch Hydrobiol* 1994;130:339–347.

57. **Chieco P, Derenzini M.** The Feulgen reaction 75 years on. *Histochem Cell Biol* 1999;111:345–358.

58. **Gupta R, Makhija S, Toteja R.** *Cell Biology Practical Manual*. Delhi, India: Prestige Publishers; 2018.

59. **Throndsen J.** Preservation and storage. In: Sournia A (editor). *Phytoplankton Manual*. Paris: UNESCO; 1978. pp. 69–74.

60. **Wancura MM, Yan Y, Katz LA, Maurer-Alcalá XX.** Nuclear features of the heterotrich ciliate *Blepharisma americanum*: genomic amplification, life cycle, and nuclear inclusion. *J Eukaryot Microbiol* 2018;65:4–11.

61. **Lessard EJ, Martin MP, Montagnes DJS.** A new method for live-staining protists with DAPI and its application as a tracer of ingestion by walleye pollock (*Theragra chalcogramma* (Pallas)) larvae. *J Exp Mar Bio Ecol* 1996;204:43–57.

62. **Strüder-Kypke MC, Montagnes DJS.** Development of web-based guides to planktonic protists. *Aquatic Microbial Ecology* 2002;27:203–207.

63. **Ruthmann A.** *Methods in Cell Research*. UK, London: G. Bell and Sons Ltd; 1970.

64. **Zhao Y, Yi Z, Gentekaki E, Zhan A, Al-Farraj SA et al.** Utility of combining morphological characters, nuclear and mitochondrial genes: An attempt to resolve the conflicts of species identification for ciliated protists. *Mol Phylogenet Evol* 2016;94:718–729.

65. **Patwardhan A, Ray S, Roy A.** Molecular markers in phylogenetic studies – a review. *J Phylogenet Evol Biol* 2014;2:1–9.

66. **Vďačný P.** Integrative taxonomy of ciliates: Assessment of molecular phylogenetic content and morphological homology testing. *Eur J Protistol* 2017;61:388–398.

67. **Tautz D, Arctander P, Minelli A, Thomas RH, Vogler AP.** DNA points the way ahead in taxonomy. *Nature* 2002;418:479.

68. **Tautz D, Arctander P, Minelli A, Thomas RH, Vogler AP.** A plea for DNA taxonomy. *Trends Ecol Evol* 2003;18:70–74.

69. **Hillis DM, Dixon MT.** Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 1991;66:411–453.

70. **Sonnenberg R, Nolte AW, Tautz D.** An evaluation of LSU rDNA D1-D2 sequences for their use in species identification. *Front Zool* 2007;4:6–12.

71. **Agatha S, Strüder-Kypke MC.** What morphology and molecules tell us about the evolution of Oligotrichea (Alveolata, Ciliophora). *Acta Protozool* 2014;53:77–90.

72. **Gao F, Gao S, Wang P, Katz LA, Song W.** Phylogenetic analyses of cyclidiids (Protista, Ciliophora, Scuticociliatia) based on multiple genes suggest their close relationship with thigmotrichids. *Mol Phylogenet Evol* 2014;75:219–226.

73. **Bachy C, Dolan JR, López-García P, Deschamps P, Moreira D.** Accuracy of protist diversity assessments: morphology compared with cloning and direct pyrosequencing of 18S rRNA genes and ITS regions using the conspicuous tintinnid ciliates as a case study. *ISME J* 2013;7:244–255.

74. **Zhao Y, Gentekaki E, Yi Z, Lin X.** Genetic differentiation of the mitochondrial cytochrome oxidase C subunit I gene in genus *Paramecium* (Protista, Ciliophora). *PLoS One* 2013;8:e77044–10.

75. **Strüder-Kypke MC, Lynn DH.** Comparative analysis of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene in ciliates (Alveolata, Ciliophora) and evaluation of its suitability as a biodiversity marker. *Syst Biodivers* 2010;8:131–148.

76. **Chantangsi C, Lynn DH, Brandl MT, Cole JC, Hetrick N et al.** Barcoding ciliates: a comprehensive study of 75 isolates of the genus *Tetrahymena*. *Int J Syst Evol Microbiol* 2007;57:2412–2425.

77. **Li J, Liu W, Gao S, Warren A, Song W.** Multigene-based analyses of the phylogenetic evolution of oligotrich ciliates, with consideration of the internal transcribed spacer 2 secondary structure of three systematically ambiguous genera. *Eukaryot Cell* 2013;12:430–437.

78. **Gao F, Katz LA, Song W.** Insights into the phylogenetic and taxonomy of philasterid ciliates (Protozoa, Ciliophora, Scuticociliatia) based on analyses of multiple molecular markers. *Mol Phylogenet Evol* 2012;64:308–317.

79. **Hoshina R.** Secondary structural analyses of ITS1 in *Paramecium*. *Microbes Environ* 2010;25:313–316.

80. **Shazib SU, Vďačný P, Kim JH, Jang SW, Shin MK.** Molecular phylogeny and species delimitation within the ciliate genus *Spirostomum* (Ciliophora, Postciliodesmatophora, Heterotrichea), using the internal transcribed spacer region. *Mol Phylogenet Evol* 2016;102:128–144.

81. **Katz LA, Deberardinis J, Hall MS, Kovner AM, Dunthorn M et al.** Heterogeneous rates of molecular evolution among cryptic species of the ciliate morphospecies *Chilodonella uncinata*. *J Mol Evol* 2011;73:266–272.

82. **Israel RL, Kosakovsky Pond SL, Muse SV, Katz LA.** Evolution of duplicated alpha-tubulin genes in ciliates. *Evolution* 2002;56:1110–1122.

83. **Roger AJ, Sandblom O, Doolittle WF, Philippe H.** An evaluation of elongation factor 1 alpha as a phylogenetic marker for eukaryotes. *Mol Biol Evol* 1999;16:218–233.

84. **Greczek-Stachura M, Potekhin A, Przyboś E, Rautian M, Skoblo I et al.** Identification of *Paramecium bursaria* syngens through molecular markers-comparative analysis of three loci in the nuclear and mitochondrial DNA. *Protist* 2012;163:671–685.

85. **Stoeck T, Przybos E, Dunthorn M.** The D1-D2 region of the large subunit ribosomal DNA as barcode for ciliates. *Mol Ecol Resour* 2014;14:458–468.

86. **Dunthorn M, Foissner W, Katz LA.** Molecular phylogenetic analysis of class Colpodea (phylum Ciliophora) using broad taxon sampling. *Mol Phylogenet Evol* 2008;46:316–327.

87. **Hebert PD, Cywinska A, Ball SL, Dewaard JR.** Biological identifications through DNA barcodes. *Proc Biol Sci* 2003;270:313–321.

88. **Stoeck T, Behnke A, Christen R, Amaral-Zettler L, Rodriguez-Mora MJ et al.** Massively parallel tag sequencing reveals the complexity of anaerobic marine protistan communities. *BMC Biol* 2009;7:72–20.

89. **Stoeck T, Bass D, Nebel M, Christen R, Jones MD et al.** Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Mol Ecol* 2010;19 Suppl 1:21–31.

90. **Huber JA, Morrison HG, Huse SM, Neal PR, Sogin ML et al.** Effect of PCR amplicon size on assessments of clone library microbial diversity and community structure. *Environ Microbiol* 2009;11:1292–1302.

91. **Vďačný P, Orsi W, Bourland WA, Shimano S, Epstein SS et al.** Morphological and molecular phylogeny of dileptid and tracheliid ciliates: resolution at the base of the class Litostomatea (Ciliophora, Rhynchostomatia). *Eur J Protistol* 2011;47:295–313.

92. Harder CB, Rønn R, Brejnrod A, Bass D, Al-Soud WA *et al.* Local diversity of heathland Cercozoa explored by in-depth sequencing. *ISME J* 2016;10:2488–2497.

93. Behnke A, Engel M, Christen R, Nebel M, Klein RR *et al.* Depicting more accurate pictures of protistan community complexity using pyrosequencing of hypervariable SSU rRNA gene regions. *Environ Microbiol* 2011;13:340–349.

94. Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM. Correction: a method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PLoS One* 2009;4:1–9.

95. Hebert PD, Penton EH, Burns JM, Janzen DH, Hallwachs W. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc Natl Acad Sci USA* 2004;101:14812–14817.

96. Mueller RL. Evolutionary rates, divergence dates, and the performance of mitochondrial genes in Bayesian phylogenetic analysis. *Syst Biol* 2006;55:289–300.

97. Hebert PD, Ratnasingham S, Dewaard JR. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc Biol Sci* 2003;270 Suppl 1:S96–S99.

98. Seifert KA. Barcoding fungi. Progress towards DNA barcoding of fungi. *Mol Ecol Resour* 2009;9:83–89.

99. Evans KM, Wortley AH, Mann DG. An assessment of potential diatom "barcode" genes (cox1, rbcL, 18S and ITS rDNA) and their effectiveness in determining relationships in *Sellaphora* (Bacillariophyta). *Protist* 2007;158:349–364.

100. Williamson P, Day JG. The problem with protists: is barcoding the answer? *Biologist* 2007;54:86–90.

101. Brunk CF, Lee LC, Tran AB, Li J. Complete sequence of the mitochondrial genome of *Tetrahymena thermophila* and comparative methods for identifying highly divergent genes. *Nucleic Acids Res* 2003;31:1673–1682.

102. Burger G, Zhu Y, Littlejohn TG, Greenwood SJ, Schnare MN *et al.* Complete sequence of the mitochondrial genome of *Tetrahymena pyriformis* and comparison with *Paramecium aurelia* mitochondrial DNA. *J Mol Biol* 2000;297:365–380.

103. Park M-H, Jung J-H, Jo E, Park K-M, Baek Y-S *et al.* Utility of mitochondrial *CO1* sequences for species discrimination of Spirotrichea ciliates (Protozoa, Ciliophora). *Mitochondrial DNA Part A* 2018;5:1–8.

104. Coleman AW. ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends Genet* 2003;19:370–375.

105. Alvarez I, Wendel JF. Ribosomal ITS sequences and plant phylogenetic inference. *Mol Phylogenet Evol* 2003;29:417–434.

106. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;31:3406–3415.

107. Miao M, Warren A, Song W, Wang S, Shang H *et al.* Analysis of the internal transcribed spacer 2 (ITS2) region of scuticociliates and related taxa (Ciliophora, Oligohymenophorea) to infer their evolution and phylogeny. *Protist* 2008;159:519–533.

108. de Rijk P, de Wachter R. RnaViz, a program for the visualisation of RNA secondary structure. *Nucleic Acids Res* 1997;25:4679–4684.

109. Gentekaki E, Lynn DH. High-level genetic diversity but no population structure inferred from nuclear and mitochondrial markers of the peritrichous ciliate *Carchesium polypinum* in the Grand River basin (North America). *Appl Environ Microbiol* 2009;75:3187–3195.

110. Finlay BJ, Esteban GF, Brown S, Fenchel T, Hoef-Emden K. Multiple cosmopolitan ecotypes within a microbial eukaryote morphospecies. *Protist* 2006;157:377–390.

111. Santoferrara LF, McManus GB, Alder VA. Utility of genetic markers and morphology for species discrimination within the order Tintinnida (Ciliophora, Spirotrichea). *Protist* 2013;164:24–36.

112. Hamsher SE, Evans KM, Mann DG, Poulíčková A, Saunders GW. Barcoding diatoms: exploring alternatives to COI-5P. *Protist* 2011;162:405–422.

113. Beszteri B, Acs E, Medlin LK. Ribosomal DNA sequence variation among sympatric strains of the *Cyclotella meneghiniana* complex (Bacillariophyceae) reveals cryptic diversity. *Protist* 2005;156:317–333.

114. Jones TC, Gates MA. A morphometric analysis of the *Euplotes charon* morphotype (Ciliophora: Euplotida). *J Eukaryot Microbiol* 1994;41:441–450.

115. Vďačný P, Slovák M, Foissner W. Multivariate morphometric analyses of the predatory ciliate genus *Semispathidium* (Ciliophora: Litostomatea), with description of *S. longiarmatum* nov. spec. *Eur J Protistol* 2014;50:329–344.

116. Lynn DH, Malcolm JR. A multivariate study of morphometric variation in species of the ciliate genus *Colpoda* (Ciliophora: Colpodida). *Can J Zool* 1983;61:307–316.

117. Vďačný P, Foissner W. A huge diversity of metopids (Ciliophora, Armophorea) in soil from the Murray River floodplain, Australia. I. Description of five new species and redescription of *Metopus setosus* Kahl, 1927. *Eur J Protistol* 2017;58:35–76.

118. Podani J. SYN-TAX. Version 5.0. *Version 5.0. Computer Programs for Multivariate Data Analysis in Ecology and Systematics*. Budapest: User's Guide. Scientia Publishing; 2001.

119. SAS Institute. *SAS OnlineDoc®, version 8*. Cary: SAS Institute; 2000.

120. Irwin NAT, Sabetrasekh M, Lynn DH. Diversification and phylogenetics of mobilid peritrichs (ciliophora) with description of *Urceolaria parakorschelti* sp. nov. *Protist* 2017;168:481–493.

121. Foissner W, Schubert G. Morphologische und diskriminanzanalytische Trennung von *Colpoda aspera* Kahl, 1926 und *Colpoda elliotti* Bradbury et Outka, 1967 (Ciliophora: Colpodidae). *Acta Protozool* 1983;22:127–138.

122. Ginoris YP, Amaral AL, Nicolau A, Coelho MAZ, Ferreira EC. Raw data pre-processing in the protozoa and metazoa identification by image analysis and multivariate statistical techniques. *J Chemom* 2007;21:156–164.

123. Maddison WP. Gene Trees in Species Trees. *Syst Biol* 1997;46:523–536.

124. Nichols R. Gene trees and species trees are not the same. *Trends Ecol Evol* 2001;16:358–364.

125. Edwards SV. Is a new and general theory of molecular systematics emerging? *Evolution* 2009;63:1–19.

126. Yang Z, Rannala B. Bayesian species delimitation using multilocus sequence data. *Proc Natl Acad Sci USA* 2010;107:9264–9269.

127. Weisse T. Functional diversity of aquatic ciliates. *Eur J Protistol* 2017;61:331–358.

128. Weisse T. Ciliates and the rare biosphere-community ecology and population dynamics. *J Eukaryot Microbiol* 2014;61:419–433.

129. Dunthorn M, Stoeck T, Clamp J, Warren A, Mahé F. Ciliates and the rare biosphere: a review. *J Eukaryot Microbiol* 2014;61:404–409.

130. Pucciarelli S, La Terza A, Ballarini P, Barchetta S, Yu T *et al.* Molecular cold-adaptation of protein function and gene regulation: The case for comparative genomic analyses in marine ciliated protozoa. *Mar Genomics* 2009;2:57–66.

131. Li J, Giesy JP, Yu L, Li G, Liu C. Effects of Tris(1,3-dichloro-2-propyl) Phosphate (TDCPP) in *Tetrahymena* Thermophila: Targeting the Ribosome. *Sci Rep* 2015;5:10562.

132. Warren A, Patterson DJ, Dunthorn M, Clamp JC, Achilles-Day UEM *et al.* Beyond the "Code": a guide to the description and documentation of biodiversity in ciliated protists (alveolata, ciliophora). *J Eukaryot Microbiol* 2017;64:539–554.

133. Ricci N. The behaviour of ciliated protozoa. *Anim Behav* 1990;40:1048–1069.

134. Banchetti R, Erra F. The ethology of protozoa and the "adaptive space" hypothesis: a heuristic approach to the biology of these eukaryotic, unicellular organisms. *Protistology* 2003;3:58–68.

135. Ricci N, Banchetti R. The peculiar case of giants of *Oxytricha bifaria* (Ciliata, Hypotrichida): a paradigmatic example of cell differentiation and adaptive strategy. *Zool Sc* 1993;10:393–410.

136. Soleymani A, Pennekamp F, Petchey OL, Weibel R. Developing and integrating advanced movement features improves automated classification of ciliate species. *PLoS One* 2015;10: e0145345.

137. Leonildi A, Erra F, Banchetti R, Ricci N. The ethograms of *Uronychia transfuga* and *Uronychia setigera* (ciliata, hypotrichida): a comparative approach for new insights into the behaviour of protozoa. *Eur J Protistol* 1998;34:426–435.

138. Bohatová M, Vďačný P. Locomotory behaviour of two phylogenetically distant predatory ciliates: does evolutionary history matter? *Ethol Ecol Evol* 2018;30:195–219.

139. Ricci N. The ethogram of *Oxytricha bifaria* (Ciliophora, Hypotrichida). I. The motile behavior. *Acta Protozool* 1981;20:393–410.

140. Ricci N, Giannetti R, Miceli C. The ethogram of *Euplotes Crassus* (Ciliata, Hypotrichida): I. The wild type. *Eur J Protistol* 1988;23: 129–140.

141. Ricci N, Verni F. The ethogram of *Litonotus lamella,* a predator ciliate. *Can J Zool* 1988;66:1973–1981.

142. Banchetti R. Ethology of Ciliates: an appreciation of the work of Nicola Ricci. *Eur J Protistol* 2003;39:380–384.

143. Wake MH. An integrative approach to the biology of biodiversity. *Bio Int* 1995;31:1–6.

144. Dayrat B. Towards integrative taxonomy. *Biol J Linn Soc Lond* 2005;85:407–415.

145. Dawson MN. Renaissance taxonomy: integrative evolutionary analyses in the classification of Scyphozoa. *J Mar Biol Ass UK* 2005;85:733–739.

146. Cedrola F, Rossi M, Dias RJ, Martinele I, D'Agosto M. Methods for taxonomic studies of rumen ciliates (alveolata: ciliophora): a brief review. *Zoolog Sci* 2015;32:8–15.

147. McManus GB, Katz LA. Molecular and morphological methods for identifying plankton: what makes a successful marriage? *J Plankton Res* 2009;31:1119–1129.

148. Buckley R. Parks and tourism. *PLoS Biol* 2009;7:e1000143.

149. Modeo L, Petroni G, Rosati G, Montagnes DJ. A multidisciplinary approach to describe protists: redescriptions of *Novistrombidium testaceum* Anigstein 1914 and *Strombidium inclinatum* Montagnes, Taylor, and Lynn 1990 (Ciliophora, Oligotrichia). *J Eukaryot Microbiol* 2003;50:175–189.

150. Rajter Lubomír, Vďačný P. Rapid radiation, gradual extinction and parallel evolution challenge generic classification of spathidiid ciliates (Protista, Ciliophora). *Zool Scr* 2016;45:200–223.

151. Sun P, Clamp J, Xu D, Huang B, Shin MK. An integrative approach to phylogeny reveals patterns of environmental distribution and novel evolutionary relationships in a major group of ciliates. *Sci Rep* 2016;6:1–12.

152. Lynn DH, Gómez-Gutiérrez J, Strüder-Kypke MC, Shaw CT. Ciliate species diversity and host-parasitoid codiversification in *Pseudocollinia* infecting krill, with description of *Pseudocollinia similis* sp. nov. *Dis Aquat Organ* 2014;112:89–102.

153. Dawson SC, Pace NR. Novel kingdom-level eukaryotic diversity in anoxic environments. *Proc Natl Acad Sci USA* 2002;99:8324–8329.

154. Dopheide A, Lear G, Stott R, Lewis G. Molecular characterization of ciliate diversity in stream biofilms. *Appl Environ Microbiol* 2008;74:1740–1747.

155. Stoeck T, Hayward B, Taylor GT, Varela R, Epstein SS. A multiple PCR-primer approach to access the microeukaryotic diversity in environmental samples. *Protist* 2006;157:31–43.

156. Puitika T, Kasahara Y, Miyoshi N, Sato Y, Shimano S. A taxon-specific oligonucleotide primer set for PCR-based detection of soil ciliate. *Microbes Environ* 2007;22:78–81.

157. Medlin L, Elwood HJ, Stickel S, Sogin ML. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 1988;71:491–499.

158. Lara E, Berney C, Harms H, Chatzinotas A. Cultivation-independent analysis reveals a shift in ciliate 18S rRNA gene diversity in a polycyclic aromatic hydrocarbon-polluted soil. *FEMS Microbiol Ecol* 2007;62:365–373.

159. Wang P, Wang Y, Wang C, Zhang T, Al-Farraj SA *et al.* Further consideration on the phylogeny of the Ciliophora: Analyses using both mitochondrial and nuclear data with focus on the extremely confused class Phyllopharyngea. *Mol Phylogenet Evol* 2017;112:96–106.

160. Hadziavdic K, Lekang K, Lanzen A, Jonassen I, Thompson EM *et al.* Characterization of the 18S rRNA gene for designing universal eukaryote specific primers. *PLoS One* 2014;9:e87624.

161. Hugerth LW, Muller EE, Hu YO, Lebrun LA, Roume H *et al.* Systematic design of 18S rRNA gene primers for determining eukaryotic diversity in microbial consortia. *PLoS One* 2014;9: e95567.

162. Xu Y, Vick-Majors T, Morgan-Kiss R, Priscu JC, Amaral-Zettler L. Ciliate diversity, community structure, and novel taxa in lakes of the McMurdo Dry Valleys, Antarctica. *Biol Bull* 2014;227:175–190.

163. Przybos´ E, Tarcz S, Surmacz M, Sawka N, Fokin SI. *Paramecium tredecaurelia*: a unique non-polymorphic species of the *P. aurelia* spp. complex (Oligohymenophorea, Ciliophora). *Acta Protozool* 2013;52:257–266.

164. Barth D, Krenek S, Fokin SI, Berendonk TU. Intraspecific genetic variation in *Paramecium* revealed by mitochondrial cytochrome C oxidase I sequences. *J Eukaryot Microbiol* 2006;53:20–25.

165. Moreira D, von der Heyden S, Bass D, López-García P, Chao E *et al.* Global eukaryote phylogeny: combined small- and large-subunit ribosomal DNA trees support monophyly of Rhizaria, Retaria and Excavata. *Mol Phylogenet Evol* 2007;44:255–266.

166. Markmann M, Tautz D. Reverse taxonomy: an approach towards determining the diversity of meiobenthic organisms based on ribosomal RNA signature sequences. *Philos Trans R Soc Lond B Biol Sci* 2005;360:1917–1924.

167. Lynn DH, Strüder-Kypke MC. Species of tetrahymena identical by small subunit rRNA gene sequences are discriminated by mitochondrial cytochrome c oxidase I gene sequences. *J Eukaryot Microbiol* 2006;53:385–387.

168. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 1994;3:294–299.

169. Zahid MT, Shakoori FR, Zulifqar S, Jahan N, Shakoori AR. A new ciliate species, *Tetrahymena farahensis*, isolated from the industrial wastewater and its phylogenetic relationship with other members of the genus *Tetrahymena*. *Pakistan J Zool* 2014; 46:1433–1445.