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ORIGINAL ARTICLE

Beyond the “Code”: A Guide to the Description and Documentation of Biodiversity in Ciliated Protists (Alveolata, Ciliophora)

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Keywords

Cultivation; information resources; molecular phylogeny; morphology; nomenclature; phylogenetics; systematics; taxonomy; type specimens.

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ABSTRACT

Recent advances in molecular technology have revolutionized research on all aspects of the biology of organisms, including ciliates, and created unprecedented opportunities for pursuing a more integrative approach to investigations of biodiversity. However, this goal is complicated by large gaps and inconsistencies that still exist in the foundation of basic information about biodiversity of ciliates. The present paper reviews issues relating to the taxonomy of ciliates and presents specific recommendations for best practice in the observation and documentation of their biodiversity. This effort stems from a workshop that explored ways to implement six Grand Challenges proposed by the International Research Coordination Network for Biodiversity of Ciliates (IRCNC-BC). As part of its commitment to strengthening the knowledge base that supports research on biodiversity of ciliates, the IRCNC-BC proposes to populate *The Ciliate Guide*, an online database, with biodiversity-related data and metadata to create a resource that will facilitate accurate taxonomic identifications and promote sharing of data.

THE nomenclature of ciliates and other heterotrophic protists (sensu Adl et al. 2012) is governed by the International Code of Zoological Nomenclature (“the Code”) that provides rules for naming taxa up to the level of family (ICZN 1999). The Code regulates nomenclatural issues, and it also establishes minimal standards for documenting newly described species. These are deliberately general and suited more to the study of animals than protists. In particular, the rule to deposit type specimens in permanently curated repositories (ICZN 1999, Art. 16.4.2 and Art. 75.3.7) has, over time, generated a database of material that not only has fixed names but also has formed the nucleus of large museum collections. These collections provide a wealth of information about the variability and

biogeography of species and represent a potential source of DNA for investigations of molecular phylogenetics.

For ciliates and many other protists, the logistical difficulties imposed by their small size meant there was no convention for depositing type specimens until the latter part of the 20th century, when deposition of permanently stained preparations or other physical specimens (e.g. on SEM stubs) began to be required as type material for taxonomic descriptions or redescriptions. In addition, new methods of visualizing morphology and sequencing genes have been introduced over the last several decades, but there are no formal standards for application of these methods to taxonomic identifications or descriptions of ciliates, despite recent recommendations (Aesch 2001,

2008; Foissner 2002; ICZN 1999; Lynn and Simpson 2009; Santoferrara et al. 2016).

In 2012, the International Research Coordination Network for Biodiversity of Ciliates (IRCNC-BC), which is jointly funded by the NSF and NSFC, identified 20 Grand Challenges of Biodiversity of Ciliates (<http://ircnc-bc.org>), of which six relate to the documentation of ciliate biodiversity:

- Understand speciation in ciliates by investigating evolutionary rates and changes in morphology, development, life cycle strategies, and genetics underlying divergence of specific lineages
- Determine the biogeographical distributions of species of ciliates, test hypotheses of endemism, and identify mechanisms and rates of dispersal
- Identify patterns of diversity and diversity "hot spots", both geographic and ecological, for ciliates
- Develop and implement a significant new paradigm for capturing and storing biological information about ciliates that can support integrative research on their biodiversity
- Establish a repository ("protist museum") to store DNA and fixed cells for use in molecular analyses
- Establish a uniform protocol for describing or re-describing species of ciliates, including minimal standards that must be met for publication and provide guidance on morphological and molecular characterization, sampling protocols, analytical methods, culturing, and long-term storage of samples.

The first three challenges are theoretical and are supported by implementation of the last three. Furthermore, the latter three challenges all relate to α -taxonomy (i.e. the description and redescription of species), the first step toward investigating biodiversity.

With this in mind, the present paper offers recommendations for using currently available methodologies and resources to generate a firm base of high-quality primary data that will facilitate and encourage modern, integrative research into the biodiversity of ciliates. The intent is to: (i) supplement guidance contained in the most recent version of the Code (ICZN 1999) for describing and redescrining species, (ii) consolidate and update recommendations for taxonomic and observational procedures, and (iii) promote the creation of information resources that will support an integrative approach to research into biodiversity of ciliates.

BACKGROUND

Historical overview

Since Antonie van Leeuwenhoek (1632–1723) made the first descriptions of ciliates (Corliss 2002; Dobell 1932), they have been discovered in almost every environment where there is sufficient water for their survival. They are common in almost all bodies of water below temperatures of about 45 °C and also live in habitats as diverse as desert sands (Foissner et al. 2002), deep-sea hydrothermal vents (Coyne et al. 2013), polar sea ice (Petz et al.

1995), and East African soda lakes (Finlay et al. 1987). Approximately 8,000 free-living and epibiotic species are recognized (Lynn 2008). Of these, roughly 4,800 are free-living, but the total is debated (Agatha 2011; Finlay et al. 1996, 2004; Foissner et al. 2008). Other ciliates are symbionts, either commensals or parasites, mostly on the external surfaces of their hosts but sometimes internally. As with free-living ciliates, the number of known symbiotic species is probably only a small fraction of their true diversity.

Toward the end of the last millennium, there was an estimated 4,300 described free-living ciliate species (Finlay et al. 1996). In the new millennium, species of free-living ciliates are being described at an average rate of ~50 per year (Foissner et al. 2008). In part, this is the result of recent explorations of poorly known or extreme environments (Agatha et al. 1990, 1993; Foissner et al. 2002, 2003; Petz et al. 1995), but a sharp increase in sampling of some understudied areas of the world, especially China and Korea, also has been a major factor. The body of morphological, environmental, and genetic information arising from such descriptions of ciliates is fragmentary relative to that of comparable-sized groups of multicellular organisms (e.g. vertebrates). This constitutes a major impediment to developing integrative uses of data from investigations of ciliates to address large-scale issues (e.g. impact of global climate change).

Over the last century, the stepwise introduction of new methods has played a major role in creating this heterogeneity of data by adding chronological "layers" of often disconnected information regarding taxa. In the 1920s–1950s, introduction of silver staining methods provided recognition of ciliary patterns and details of nuclear morphology (Chatton and Lwoff 1930; Cole and Day 1940; Fauré-Fremiet 1950; Klein 1926); these observations allowed better α -taxonomy and stimulated major revisions of ciliate taxonomy and classification (Corliss 1961; Janakowski 2007; Lynn 2008). Beginning in the 1950s, application of electron microscopy to investigate organelles and ultrastructures added another "layer" of data. The rapid development of PCR and gene sequencing during the past three decades fueled a leap in our ability to address taxonomic and phylogenetic questions about ciliates. However, they also contributed to the heterogeneity of data by promoting separate paths of training: molecular vs. morphological. Finally, information has been gathered on the ecophysiology, biogeography, and behavior of ciliates, which has been used to complement morphological, morphogenetic, and molecular characterization of taxa. Although all of the above methods continue to be applied to ciliate taxonomy, there have been few attempts to rationalize and combine their application.

The species concept applied to ciliates

As with other organisms, it has been difficult to settle on an acceptable definition of what constitutes a species of ciliate. Sonneborn (1957) suggested the "principle of minimal irreversible evolutionary divergence". Although

“irreversible” may no longer be appropriate as reversals over evolutionary distance occur (Seifert 2014), Soneborn’s concept can be amended to be consistent with α -taxonomy by deleting “irreversible” (Schlegel and Meisterfeld 2003). Molecular, morphological, or other characters can then be used as evidence of evolutionary divergence, an approach that has been termed the “Pragmatic Species Concept” (Seifert 2014). Insofar as ciliate morphology is closely related to the function of the organism in nature, the morphospecies concept is as valid as any, and probably more pragmatic than any other. Finlay et al. (1996) defined the morphospecies as “a collection of forms that all fit into a defined range of morphological variation—forms that, so far as we can tell, occupy the same ecological niche”, and concluded that “at present, it is difficult to see any other method of differentiating and identifying free-living ciliates”. Thus, the morphospecies concept is the one most widely adopted in the absence of any pragmatic, viable alternative.

Documenting and accessing data relating to biodiversity of ciliates

The goal of integrative biodiversity has been defined as delimiting the units of life’s diversity from multiple and complementary perspectives such as phylogeography, comparative morphology, population genetics, ecology, development, and behavior (Dayrat 2005). There is, however, anecdotal evidence of a decline in taxonomic expertise in most countries of the world. This “taxonomic impediment” (de Carvalho et al. 2007) is a concern in respect to all eukaryotes and specifically threatens progress in ciliate biodiversity and evolution. At the same time, the exponential increase in metagenomics and environmental sequence data, combined with the development of methods for sharing information via the Internet and in open-access journals, is providing opportunities for progress toward documenting ciliate biodiversity. One increasingly popular strategy for streamlining the formal description of larger numbers of new species combines concise morphological descriptions by expert taxonomists with DNA barcodes and high-resolution digital imaging. The term “turbo-taxonomy” has been coined for this approach (Butcher et al. 2012), which, however, might cause serious problems in the future. Consequently, there is a need to move toward closing the gaps in the taxonomic knowledge of ciliates. This will require a solid “systematics portal” to act as a foundation to make information (e.g. digitized images of type specimens, barcodes, and diagnoses) more accessible (Godfray 2007).

Progress with the Grand Challenges of Biodiversity of Ciliates hinges on both describing species remaining to be discovered and on redescribing the many species for which only scanty descriptions exist. Researchers require cross-training in morphological and molecular methods and an understanding of ecological and behavioral aspects. Data must then be made available through systems for sharing, archiving, and searching taxonomic information (i.e. ones that are efficient, permanent, cost-effective, and freely available world-wide). In the following sections, we

review key issues of taxonomic procedures as they relate to ciliates and recommend standards for generating the foundation of data needed for the integrative investigations of their biodiversity. These will be categorized as ones that **MUST** be done to satisfy basic requirements for taxonomic identification or description of ciliates, ones that **SHOULD** be done to ensure that the highest quality of taxonomic data are presented, and ones that **COULD** be done to maximize the value of data to integrative investigations.

TAXONOMIC PROCEDURES

Rules of nomenclature

Nomenclatural acts relating to taxa with ranks from subspecies to superfamily *must* follow the Code (ICZN 1999; <http://iczn.org/iczn/index.jsp>), which includes amendments (ICZN 2012) addressing issues associated with publication practices (inclusive of electronic-only publications) and the use of a registry (ZooBank). Concerns over editorial competence in taxonomic matters and nomenclature for certain electronic-only journals (Dubois et al. 2013) suggest that taxonomic work *should* be published in journals that print at least some multiple identical paper copies, unless they have a track record for handling taxonomic issues (i.e. major journals, published for many years). For taxonomic papers where longevity is required, taxa *should* be published in electronic-only journals only if the journal has an affiliation with an archive that meets the requirements of Article 8.5.3.1 of the 2012 Code amendment (ICZN 2012). An example of such an archive is CLOCKSS (<https://www.clockss.org/>) which guarantees to make archived journal files available should the publisher be unable to continue supporting the journal. All nomenclatural acts *should* be added to ZooBank (Pyle and Michel 2008), the official registry of zoological nomenclature maintained by the ICZN (<http://iczn.org/content/about-zoobank>). For further discussion on the utility and application of the Code, see Dubois et al. (2016) and Dubois and Aescht (2016).

Key issues not regulated by the Code include what criteria define species- to family-level taxa (see section on species concept above), taxa above family rank, and phylogenetic elements. The following *should* be consulted: Corliss (1972) provides practical guidelines for higher taxa of ciliates; Aescht (2001, 2008) provides a checklist of required and recommended procedures for complying with provisions of the Code when describing or redescribing species or genera of ciliates.

A type specimen is only the name-bearer of a nominal species. Therefore, it *should not* be considered as source of all descriptive data needed in (re)identification, or documenting the range of variation of a species. While the type specimen is a nomenclatural concept, the (re)description refers to taxonomic functions. This is the main reason why the possibility of designating one or more hapantotypes *should not* be used unless there is a compelling reason for doing so, e.g. specimens at different stages in a life cycle. Although not a strict requirement of the Code, type specimens *must* be deposited in permanently curated

repositories (e.g. museums), not in private or temporary collections. If name-bearing types are deposited in a laboratory collection, there *must* be a formal agreement, cited in the original description along with the name of a secondary repository that specifies provisions for permanent curation by the institution in which the laboratory is housed and for passing types to the secondary institution if curation cannot be maintained. Paratype specimens *should* be placed in a different repository than the one that houses the holotype if it is deposited in any collection other than one in a major museum. Voucher specimens and associated data *should* be deposited when they are used for taxonomic works (e.g. revision of taxa); where possible this practice *could* be applied to nontaxonomic works where accurate identification of species may be important (e.g. some ecological investigations). A voucher specimen is defined as a nominated specimen representing the taxonomic unit mentioned in a work, which may or may not be any category of type, e.g. a specimen used in a physiological study or retained as evidence that it was found in a particular location (Hawksworth 2010). Two reprints or a pdf copy of the paper in which type or voucher specimens were designated *should* be sent to each repository, to preserve the link between specimen(s) and nomenclatural acts.

Composing descriptions and redescrptions

Winston (1999) is an invaluable guide to describing species and *should* be followed, as descriptions and redescrptions of ciliates should conform to standards applied to other eukaryotes. Recent descriptions and redescrptions of ciliates published in major journals of protistology *should* be consulted for guidance in formatting and inclusion of data. Descriptive terms *must* follow those currently accepted (for a glossary, see Lynn 2008), and their usage *must* be consistent within the description (i.e. avoid synonyms). An analysis of gene sequences *should* be included in a description of a new species or redescription of a known species, unless material that can be used for extraction of DNA is unavailable. If it is not possible to perform a molecular analysis, cells *should* be deposited in a publicly accessible repository. In fact, generally, for all type depositions, material *should* be deposited for later molecular analysis (Dayrat 2005).

Making accurate identifications

Many ciliates are known from only a short original description in the older literature, which is an impediment to accurate identifications. Comprehensive taxonomic sources exist only for few groups of ciliates (e.g. Berger 1999; Vd'ačný and Foissner 2012). Therefore, identifications of known ciliate species *should* be supported by detailed comparisons with the original descriptions that confirm its diagnostic features and add new information. If the specimens studied match only some of the published illustrations, these *should* be itemized. If a redescription is used instead of the original description, this *should* be justified. Establishment of new species *should* be

accompanied by a justification for the generic affiliation and a comparison with morphologically similar species.

Depositing type and voucher specimens in collections

Stained permanent slides upon which descriptions of novelties are based and redescrptions where a neotype is designated *must* be deposited (Aescht 2008; Lynn and Simpson 2009). These slides *should* constitute a homogeneous sample of the specimens used for the work and not specimens collected at different places or at different times whose conspecificity is uncertain although a heterogeneous sample might be designated as a syntype under the rules of the Code (Art. 73.2). One or more preparations of directly related individuals, e.g. representing different stages in the life cycle, may be designated as a hapantotype, which is the holotype of the nominal taxon (Art. 72.5.4 and Art. 73.3). The continued acceptance of the hapantotype concept, however, has recently been questioned (Dubois et al. 2016).

Permanent preparations *must* conform to the basic definition of a type specimen [for type definitions, see International Commission for Zoological Nomenclature (1999) and Aescht (2008)] by revealing sufficient diagnostic characters to allow unambiguous identification and, therefore, *should* be fixed and stained by a method that shows taxonomically critical features of the major taxon of ciliates to which the species belongs. At present, the two methods that fulfill these criteria most reliably are protargol and silver nitrate (Foissner 2014), although others may become appropriate. Some staining methods (e.g. ammoniacal silver carbonate) result in samples that fade and *should not* be used to prepare type specimens. Type slides cannot be a source of DNA for sequencing, so additional cells *should* be deposited for future analysis (e.g. in 95% alcohol at -20°C or cryopreserved).

In general, voucher material representing populations examined for special purposes (e.g. DNA barcoding, functional ecology) *should* be submitted to a repository for long-term preservation. This has not been a common practice for ciliates, with the consequence that much of the ecological literature contains taxonomic identifications that cannot be verified. At a minimum, vouchers *could* consist of photomicrographs or videos displaying the taxonomically relevant features plus measurements of the organisms. For documenting barcodes, preserved cells showing the taxonomically relevant structure *should* be made available. Several of the world's major museums, universities, and research institutes hold publicly accessible ciliate collections, of which the following are especially notable.

- Biology Centre of the Upper Austrian Museum, Linz, Austria (<http://www.landesmuseum.at/>): approximately 3,500 slides (holotypes, syntypes, hapantotypes, neotypes, or paratypes) of 990 species representing approximately 420 genera and 150 families; about 20,000 voucher slides of protists
- Laboratory of Protozoology, Institute of Evolution and Marine Biodiversity, Ocean University of China, Qingdao, China (<http://www.ouc.edu.cn/english/>);

about 2,500 slides of 830 nominal species, including 420 type slides

- International Protozoan Type Slide Collection, Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012, USA (<http://invertebrates.si.edu/collections.htm>); 518 items deposited as type material (470 microscope slides, six SEM stubs, 42 alcohol- or formalin-preserved specimens) representing 119 holotypes, 135 paratypes, 34 lectotypes, 34 paralectotypes, 10 hapantotypes, 28 syntypes and 111 vouchers of more than 350 nominal species
- Natural History Museum, London, U.K. (<http://www.nhm.ac.uk/>); approximately 500 slides (holotypes, hapantotypes, neotypes, paratypes, or vouchers) (<http://www.nhm.ac.uk/research-curation/research/projects/protists/>), about 22,000 slides of other protists and over 1,000 video sequences of protists (<http://www.nhm.ac.uk/research-curation/research/projects/protistvideo/>)
- Muséum National d'Histoire Naturelle, Paris, France (<http://www.mnhn.fr/fr/>); 114 type slides, most of which were collected in France
- Zoological Survey of India, Kolkata, India (<http://www.zsi.gov.in/>); approximately 1,130 slides, 49 holotypes and 104 paratypes
- Australian Museum, Sydney, Australia (<http://australianmuseum.net.au/>); 59 lots of registered specimens, mainly epibiotic species from Australian freshwater crustaceans preserved in 70% ethanol but also including 13 type slides
- National Marine Biodiversity Institute of Korea, Chungcheongnam-do, Korea (<http://www.mabik.re.kr/>); slides of approximately 80 marine species, including 12 type slides (1 holotype and 11 paratypes) of ten species.

DEPOSITION, CONSERVATION, AND CURATION OF ANCILLARY MATERIAL

General considerations

Beyond depositing material as outlined above, researchers can make supplementary material and data available to future investigators and the public. There are three major issues that must be considered when providing such ancillary materials. They *should* be:

- Provided and maintained with forethought to the development of new technologies or methodologies
- Offered by the curation facility in a form that will be available at no cost to stakeholders
- Of sufficient quantity and quality to be of utility.

Photomicrographs, videos, or line drawings of cells

Type and voucher slides are useful to taxonomic specialists but are not readily accessible to other potential users, e.g. those not listed as "approved users" of certain museum collections. This issue *could* be addressed by

making high-resolution digital images of the specimen(s) accessible. Best practice would be to provide images of the specimen(s) taken at different focal planes to allow assembly into 3-D composites. Such images may then be adaptable to more sophisticated future methodologies, in which physical type or voucher specimens will be accompanied by deposition of raw and 3-D holographic images in databases (e.g. GBIF).

Here, we suggest that a single portal would facilitate a variety of integrative investigations, allowing newly collected material to be compared with type and voucher specimens, using bioinformatic and image-resolution software or dedicated search engines. At present, this kind of ability is being created mainly for molecular data; however, morphological, morphogenetic, behavioral, or physical data have their own intrinsic value in terms of revealing ecological or physiological functionality and will become increasingly important as integrative approaches to the investigation of biodiversity continue to evolve.

Molecular specimens

Molecular data are now used in virtually all areas of biological research. Consequently, major natural history museums have established facilities to house collections of physical specimens that can be used in molecular investigations. Prominent among these are the American Museum of Natural History, New York (<https://research.amnh.org/amcc/>), the Smithsonian National Museum of Natural History, Washington, DC (<http://www.mnh.si.edu/rc/biorepository/index.html>), the Muséum National d'Histoire Naturelle, Paris (<https://www.mnhn.fr/fr/collections/ensembles-collections/ressources-biologiques-cellules-vivantes-cryoconservees>), the Natural History Museum, London (<http://www.nhm.ac.uk/our-science.html>), and the Museo Nacional de Ciencias Naturales, Madrid (<http://www.mncn.csic.es/>). These house permanently curated repositories for samples of frozen material and environmental samples that are maintained under controlled conditions to make them readily accessible to researchers. Molecular specimens are given an identifier, such as a registration or accession number, allowing further genetic analyses. In this respect, they are similar to traditional museum specimens.

Reliable methods of preserving cells or extracted DNA and RNA that have been applied to ciliates (either as samples of whole cells or within an environmental sample) are FTA Cards (Whatman/GE Healthcare Life Sciences, Pittsburgh, PA; Hide et al. 2003) and RNAlater® (Ambion, Carlsbad, CA). Another method potentially for use with ciliates is DMSO/EDTA/saturated NaCl (DESS) (Gray et al. 2013). The range of materials that can be stored in natural history repositories and that could be used for ciliates includes: (i) specimens deep-frozen in liquid nitrogen at $-196\text{ }^{\circ}\text{C}$; (ii) whole cells stored at $-80\text{ }^{\circ}\text{C}$; (iii) alcohol-preserved specimens stored at $-20\text{ }^{\circ}\text{C}$; (iv) DNA extracted from nuclei and mitochondria stored in water or buffers at $-20\text{ }^{\circ}\text{C}$; (v) silica-dried material stored

at room temperature; (vi) extracted RNA stored at -80°C ; (vii) genomic phage/BAC libraries stored at -80°C ; (viii) expression (cDNA) libraries in bacterial culture; (ix) DNA/RNA samples and expression (cDNA) libraries on FTA cards stored at room temperature; and (x) lyophilized/freeze-dried material stored at room temperature.

Molecular data and analyses

Although not regulated by the Code, additional deposition of genetic information in publicly available databases (e.g. NCBI GenBank) *should* be included in a description or redescription (e.g. Lynn and Simpson 2009). Most individual sequences that have been deposited are the gene coding for the small subunit ribosomal DNA (SSU rDNA), which is useful for ciliate identification and phylogenetic analysis. Currently, there are SSU rDNA sequences linked to approximately 850 named species of ciliates (Fig. 1). This represents only $\sim 10\%$ of the known ciliate species (Lynn 2008). Many of these are associated with deposited material, but most are not associated with physical specimens of any sort.

To date, there is no easily searchable database for housing analyses of data such as alignments and phylogenetic trees. TreeBASE (<http://treebase.org/treebase-web/about.html>), intended to fill this role, is not searchable by taxonomic category or using names of species (Anwar and Hunt 2009). It is possible to link data to a publication as supplementary material, but this will scatter information among sources that are not easily searched. Hence, we suggest that the online *Ciliate Guide* (<http://ciliateguide.myspecies.info/>) *should* be used, where data can be linked

to names of species or higher order taxa in a searchable database. Analytical material such as nexus files of alignments or Newick files of phylogenetic trees also *should* be deposited in TreeBASE, as it provides quality control (e.g. nexus files of alignments must be capable of being opened in Mesquite) and updating of information by curators.

Genetic data must be evaluated in context of morphological characters to address broad questions about complex processes that involve multiple factors such as evolutionary rates, convergent evolution, population structure, and functional ecology acting in concert. This is also true for relatively narrow avenues of inquiry such as α -taxonomy. For example, the degree of divergence between sequences of a key gene (e.g. SSU rDNA, ITS, CO1), by itself, cannot substitute for actual characters because there is no generally accepted threshold value for the degree of divergence between congeneric species, including cryptic and pseudocryptic species or higher taxa. Analysis of DNA from environmental samples reveals a large and hidden diversity of ciliates that is rarely reported in morphological surveys of ecosystems studied so far.

Cultures and preserved viable cells

Research into some aspects of ciliate biodiversity is constrained by the difficulty of establishing and maintaining them in long-term cultures or preserving cells in a viable state. Thus, unlike other microorganisms (e.g. bacteria, algae, fungi), there is no tradition for designating viable protozoan cells preserved in a metabolically inactive state or protozoan cultures as a name-bearing type. Given the small number of ciliate strains in stable cultures and the technical difficulty of maintaining live cultures or preserved viable cells, it is premature to suggest a similar role for cultures of ciliates. In a few cases, however, long-term maintenance of ciliate cells in an inactive but viable state has been achieved by cryopreservation (Daggett and Nerad 1992; Müller et al. 2008, 2010; Nerad and Daggett 1992; Simon and Hwang 1967). Also, few ciliate cultures (~ 700 strains belonging to 54 genera) are deposited in culture collections that accept protists (American Type Culture Collection/ATCC; www.lgcstandards-atcc.org/ and Culture Collection of Algae and Protozoa/CCAP; www.cca.p.ac.uk), from which they are available as live cultures or cysts. Most cultured species are members of a few genera used extensively as model organisms (e.g. *Tetrahymena*, *Paramecium*, *Euplotes*). We recommend that researchers *could* isolate and deposit cultures of taxa they describe, and the following resources are available to this end: (i) formulations for media and protocols for starting and maintaining cultures (Day et al. 2007; Finlay et al. 2000; Lee and Soldo 1992; Nerad 1991; Tompkins et al. 1995); (ii) protocols for preserving dried cysts on filter paper (McGrath et al. 1977); and (iii) protocols for cryopreservation (Müller et al. 2008).

Biological Resource Centers (BRCs), such as the ATCC or the CCAP, play a key role in safeguarding and providing cultures, with the main objective being the preservation of

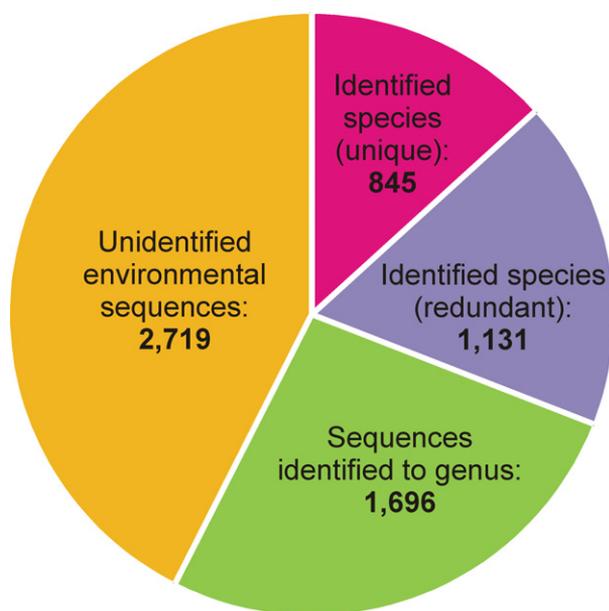


Figure 1 Ciliate sequences of the small subunit ribosomal RNA gene in the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>; accessed on 6/4/2015).

the phenotypic and genotypic characteristics of the conserved taxa (Müller et al. 2008). In recent years, the role of BRCs has been extended, largely as a result of the rapid increase in metagenomics and environmental sequencing data. The CCAP has responded to this challenge by developing KnowledgeBase, for quality-controlled biological material, hyperlinked to manually curated molecular, bibliographic, and taxonomic information (Gachon et al. 2013).

MORPHOLOGICAL AND ONTOGENETIC CHARACTERS

Documenting morphology and ontogeny

Virtually the entire body of knowledge about ciliates is linked to morphospecies. Therefore, morphological characters *must* be studied for species descriptions and redesignations and *should* be included with other characteristics when investigating aspects of ciliate biodiversity. Best practice *should* be to measure, count, describe, and illustrate every observable detail of morphology and ontogeny, even though all data might not be published. Where known cryptic species complexes have been recognized by molecular characters (e.g. isozymes, *cox1*, ITS2; Nanney and McCoy (1976), Sonneborn (1957) and references in section below on DNA barcoding) a detailed morphological description *should* be carried out with the aim to search for new taxonomically relevant characters that can be analyzed by multivariate statistics (e.g. Gates and Berger 1974, 1976; Powelson et al. 1975).

If possible, clonal cultures *should* be used for observations to avoid reliance on data from field samples alone that create the risk of confusing morphologically similar species. Some species also have polymorphic life cycles that only can be revealed under culture conditions, including using different conditions (e.g. *Tetrahymena aquasubterranea*; Quintela-Alonso et al. 2013). However, it should be noted that cells in culture may exhibit phenotypes not typically seen in nature, so culture conditions *must* be described.

A description or redesignation *must* be based on deposited, fixed material, revealing the taxonomically relevant characters and also *must* be based on live observation (Foissner 2014), except when meeting either or both of these requirements is not reasonable and other material (physical or virtual) allowing a reinvestigation of the diagnostic features is deposited. Cells can shrink or swell during fixation, and the characters of some taxa can contract and/or disappear; therefore, live observations provide useful information (e.g. see Fig. 2). Information obtained from live observation *should* include:

- Cell size and shape
- Cilia lengths (oral, somatic, brush, etc.)
- Cytoplasm color
- Contractility and flexibility of cell
- Presence, color, and arrangement of cortical granules
- Presence of symbiotic algae, bacteria, or sequestered plastids

- Presence, size, shape, and location of mature extrusomes
- Presence, position, number, and shape of contractile vacuoles and the contractile vacuole pore
- Composition, shape, and location of the nuclear apparatus.

Information obtained from live observation *could* also include:

- Variations in cell size and shape associated with division, life cycles, or environmental conditions
- Position and shape of the cytophyge
- Presence of lipid droplets, crystals, or other inclusions
- Contents of food vacuoles (indicating the role of the organism in the microbial food web—with the caveat that the types of food eaten may vary according to local conditions and may interact with abiotic factors to affect morphology or activity).

At present, staining with protargol or silver nitrate *must* be performed to provide fixed type material but where this is not reasonable, equivalent methods revealing the diagnostic features *must* be applied providing permanent type material (physical or virtual). Silver carbonate stains may be used to augment descriptions, but these fade and their results *must* be documented photographically (Fig. 2). In the future, it *could* be more appropriate to apply fluorescent staining of structures to augment descriptions (Arregui et al. 2002), enabling detailed examination by confocal microscopy. Information recorded from silver-stained preparations *should* include:

- Pattern of somatic ciliature to include general configuration, number, and orientation of kineties; number and spacing of kinetids in kineties, and structure of kinetid and brush (if present)
- Pattern of oral ciliature (i.e. arrangement, number, and structure of ciliary organelles)
- Structure of nuclear apparatus (i.e. number, shape, size, and location of nuclei and shape of nucleoli)
- Miscellaneous features, such as positions and appearance of organelles (e.g. argyrophilic granules and fibrillar structures).

There is a range of other features that also may be examined. Ontogenesis (i.e. cell division including stomatogenesis, morphogenesis, and the regeneration of parental structures) *should* be included in descriptions when it provides taxonomically relevant features, e.g. the position of the oral primordium is diagnostic in oligotrichid ciliates (Agatha 2004) and the lack of a frontoventral cirrus in a certain position is informative in euplotids (Borror and Hill 1995); otherwise, the investigation of this process is recommended as even in well-known ciliate groups, new patterns of cell division may be discovered (e.g. in halteriids by Song 1993). Resting cysts, if present, *should* be examined as morphological diversity occurs in some morphospecies, which may suggest the existence of pseudocryptic species (Foissner et al. 2002; Katz et al. 2005; Xu and Foissner 2005). Mating type interactions *could* provide insights into the breeding

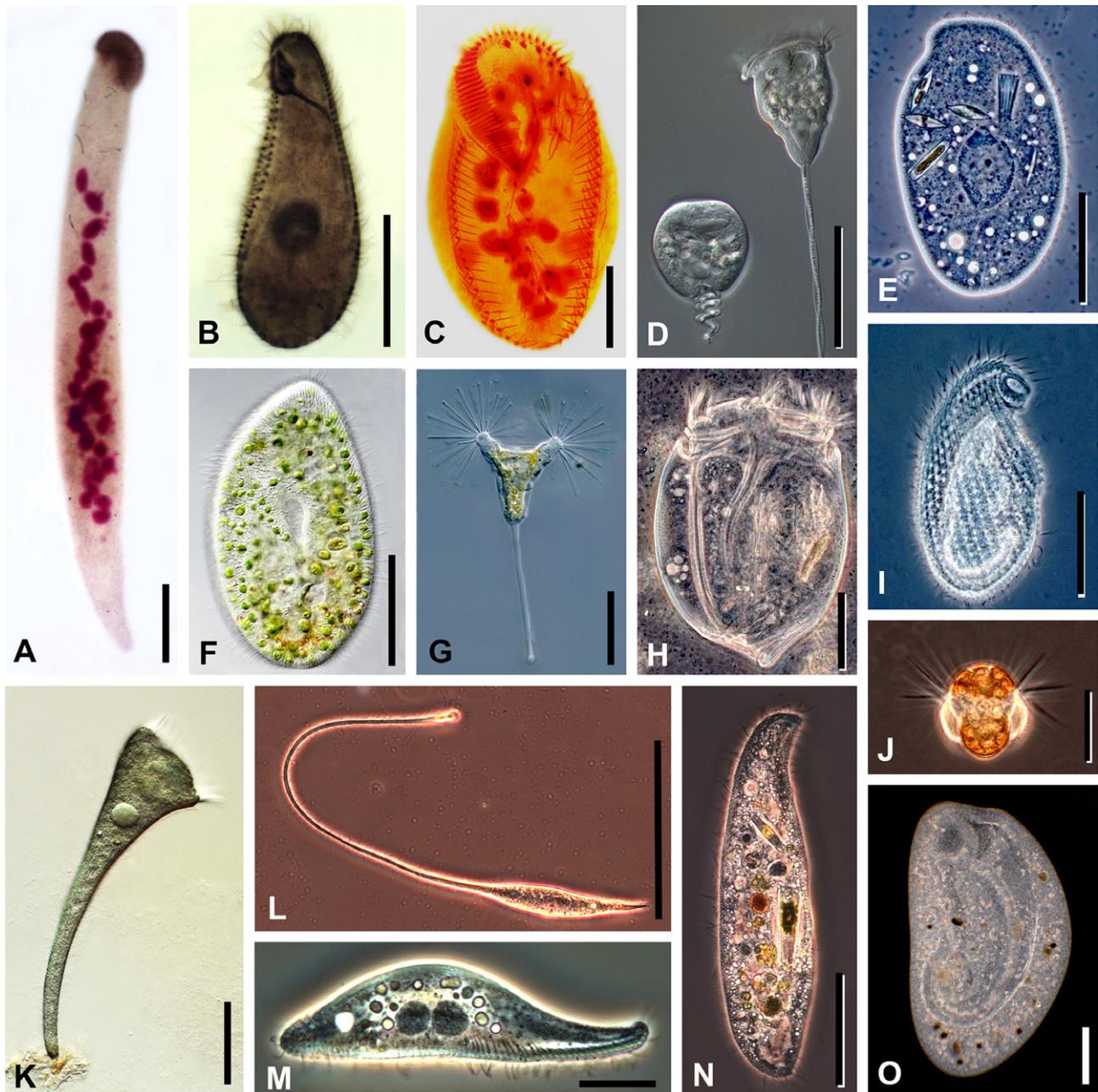


Figure 2 Photomicrographs of representative ciliate taxa (originals from D. J. Patterson). **(A)** *Homalozoon vermiculare*, a crawling, ribbon-shaped haptorian predator. The cell has been fixed and stained with Feulgen reagent. The anterior mouth, at the top of the image, is underlain with a dense band of extrusomes that are used to capture food. The structures stained pink are the irregular ribbon of macronuclear nodules, the micronuclei, and a few developing extrusomes. Scale bar = 50 μm . **(B)** *Tetrahymena pyriformis*, a hymenostome ciliate. The cell has been fixed and stained with protargol that impregnates the bases of the cilia (basal bodies) and the nucleus. The somatic cilia are arranged in longitudinal rows; the oral cilia are densely packed and appear as short bands. A system of microtubules that forms the cytopharynx extends from the mouth into the cell. The macronucleus is the globular structure near the center of the cell. Scale bar = 20 μm . **(C)** *Pattersoniella vitiphila*, a hypotrich ciliate. Fixed cell stained with protargol. The C-shaped collar around the front (top) of the cell is the adoral zone of membranelles (ciliary fans). The spots near the front of the cell, forming marginal bands and a curving row in the center of the cell, are cirri (ciliary bristles). The macronuclear nodules are the globular inclusions within the cell. Scale bar = 50 μm . **(D)** *Vorticella convallaria*, a peritrich ciliate. Two living cells under Nomarski differential interference contrast optics, the one to the right is extended and feeding, the one to the left is contracted. The feeding cilia (haplokinety and polykineties) extend around the edge of the anterior of the cell and curve into the mouth. The cells contain many food vacuoles. The stalk contains a spasmoneme that causes it to contract in a spiral fashion, while an elastic envelope acts as antagonist. Scale bar = 50 μm . **(E)** *Trithigmostoma* sp., a cyrtophorid ciliate. Living cell under phase-contrast optics. The cell eats small algae, using the basket-shaped mouth (upper right). Several ingested diatoms can be seen inside the cell.

The large central structure is the macronucleus. There are multiple small contractile vacuoles in the cell that are used for osmoregulation. Scale bar = 50 μm . (F) *Paramecium bursaria*, a peniculiid ciliate with endosymbiotic zoochlorellae. Living cell under Nomarski differential interference contrast optics. The cell surface is underlain with many rod-shaped extrusomes (minute harpoon-like structures). The pear-shaped area in the center of the cell is the cytopharynx. Scale bar = 50 μm . (G) *Acineta* sp., a suctorian ciliate. Living cell under Nomarski differential interference contrast optics. This predator captures other ciliates and flagellates that swim into the extending sticky tentacles. Each tentacle is a mouth and has an expanded apex containing extrusomes that catch hold of any prey. The cell is attached to substrates by means of a stalk. Cilia are absent in the trophic cell, but present in the swimmers. Scale bar = 50 μm . (H) *Eudiplodinium* sp., an entodiniomorphid ciliate from the stomach of a bison. Living cell under phase-contrast optics. The body is very stiff, sculpted with longitudinal grooves that form a rounded extension at the posterior end of the cell. There are two zones of cilia at the anterior end. The ciliate feeds on bacteria, many of which can be seen around the cell, and on small particles of wood. Two contractile vacuoles and associated vesicles lie over the macronucleus in the left cell half. Scale bar = 20 μm . (I) *Cryptopharynx* sp., a small and rarely encountered karyorelictean ciliate. Living cell under phase-contrast optics. The mouth is the extension to the anterior right. The cilia form dimpled rows (kineties) that extend along the length of the body. Scale bar = 20 μm . (J) *Mesodinium rubrum* (syn. *Myrionecta rubra*), a small marine planktonic haptorian ciliate that contains endosymbiotic cryptomonad algae giving the cell its red color. Living cell under phase-contrast optics. Stiff bundles of equatorial cilia extend from the cell and their action causes a jumping motion; further cilia lay adpressed to the posterior portion of the body. Scale bar = 20 μm . (K) *Stentor coeruleus*, a trumpet-shaped heterotrich ciliate. Living cell under Nomarski differential interference contrast optics. The cell attaches to immersed surfaces via its posterior end and, when feeding, the cell has a characteristic conical form with the adoral zone of membranelles at the anterior of the cell. Cilia also extend from the remaining body surface. The blue color comes from minute inclusions (cortical granules) lying just under the cell surface. Scale bar = 100 μm . (L) *Lacrymaria olor*, a predatory haptorid ciliate. Living cell under phase-contrast optics. The extensible and contractile anterior portion moves around, searching for prey, while the posterior end of the body often remains attached to the substrate. The mouth is at the tip of the extensible neck and is expanded because of the extrusomes that are used to kill prey—often small ciliates. Scale bar = 50 μm . (M) *Litonotus* sp., a pleurostomatid predatory ciliate. Living cell seen from the side under phase-contrast optics. There are two large macronuclear nodules in the center of the cell. The mouth lies along the bottom edge of the cell, extending from near the middle to the anterior end, and is underlain with extrusomes that are used to kill prey. Somatic cilia extend from the ventral surface of the cell. Scale bar = 20 μm . (N) *Loxodes* sp., a karyorelictid ciliate from freshwater habitats. Living cell under phase-contrast optics. The sickle-shaped mouth region is located to the anterior right of the cell, which engulfs mostly algae, such as diatoms, and cyanobacteria. One of the macronuclear nodules is evident just above the mid-line and in the left cell half; it has a dark core and a light peripheral region. Anteriorly and posteriorly to it are two vacuoles of a similar size but each with a bright refractile granule. These are Müller's bodies that inform the cell as to the direction of gravitational pull. Scale bar = 50 μm . (O) *Bursaria* sp., a huge colpodid ciliate. Living cell under dark field optics. There is a large, curved channel that leads from the anterior of the cell to the cytostome. The ciliate is mostly found in still-freshwater habitats, where it feeds on planktonic algae. Scale bar = 50 μm .

structure of a species (Nobili et al. 1978). Finally, some taxa (e.g. dileptids; Vd'ačný and Foissner 2012) display a regenerative capacity that *should* be documented.

Illustrating morphology

Observations that form the basis of descriptions or redescriptions *must* be documented by illustrations and *should* be accompanied by photomicrographs. A useful source regarding techniques to produce illustrations acceptable for publication is the Guild of Natural Science Illustrators (<https://gnsi.org/>). The handbook of the Guild (Hodges 2003) is the definitive source, but there are others (Ridgway 1978; Wood and McDonnell 1994; Zweifel 1988). Silver-stained preparations *should* be used as the primary basis of illustrating specimens with line drawings. As photomicrographs of silver-stained specimens show only one focal plane of the cell, image-stacking software *could* be used to assemble several planes into a single composite image. Fluorescently labeled taxoids such as FLUTAX (Santa Cruz Biotechnology Inc., Dallas, TX) that bind specifically to microtubules *could* also be used, possibly in conjunction with confocal microscopy, to assess patterns of ciliature and infraciliature during the identification process (Arregui et al. 2002). The style for the arrangement of illustrations and plates, use of standard abbreviations, and other stylistic conventions *should* follow those in the recent literature.

Morphometric data

Data obtained by morphometric studies *must* be derived from a sufficient number of appropriately oriented morphostatic specimens to provide information on the range of the characters and allow the species to be separated from other closely related taxa; this will vary depending on the taxon and the feature, but between 10 and 30 measurements *should* generally be appropriate. Data *should* be presented as standard descriptive statistics (e.g. mean, standard error, range). However, as measurements not provided in the description may emerge as diagnostic, the raw data *should* be submitted as supplementary material.

MOLECULAR CHARACTERS

Using molecular data as taxonomic characters

Molecular phylogenies that disagree with classifications based on morphological characters create debate regarding the relative merits of these approaches, but in doing so they initiate the search for new characters. Certainly, the new molecular technologies cause similar large-scale readjustments in the classification of ciliates like the previous introductions of new methods for studying morphological features. Molecular evidence is still relatively new, and the technology for obtaining it is evolving rapidly;

therefore, pitfalls and limitations continue to emerge. Accordingly, use of molecular characters by themselves for defining taxa must be done with caution and only if every attempt to relate other features (described above) with molecular characters failed. There are already precedents for this, both in ciliates and in other groups of protists (Dunthorn et al. 2014; Hoef-Emden and Melkonian 2003; Huss et al. 1999; Marin et al. 2003; Pröschold et al. 2001; Sun et al. 2012; Zhan et al. 2009).

In practice, molecular characters are not materially different from other categories of evolutionary evidence because they are constrained by the same analytical criteria. They *must* be evaluated critically like other characters (i.e. a priori vs. a posteriori weighting) and *should* be used to formulate phylogenetic hypotheses that can be tested by enlarging the dataset with further morphologic and molecular characters and taxa (Neff 1986). As with other sorts of characters, reliability of molecular characters *must* be evaluated by a thorough comparison within and between taxa and with cladistic analyses to discover elements such as homoplasies or polymorphisms, with the goal of detecting robust synapomorphies. Finally, molecular characters *must* be described, illustrated, and discussed.

DNA barcoding

Protists are so genetically diverse owing to their long independent evolutionary histories that it has been impossible to identify a single DNA barcode for all of them. Consequently, the Consortium for the Barcode of Life Protist Working Group (CBOL ProWG) was established in 2012 to identify standard barcode regions for protists and to assemble a reference library (Pawlowski et al. 2012). The CBOL ProWG suggests a two-step barcoding approach of preliminary identification: a universal eukaryotic barcode (the pre-barcode) followed by a species-level assignment using a group-specific barcode. Based on comparative studies and applying the CBOL (<http://www.barcodeoflife.org/>) selection criteria, the ProWG suggests the ~500 bp long variable V4 region of the 18S rDNA as the universal pre-barcode, with group-specific barcodes then being defined separately for each major protistan group (Pawlowski et al. 2012).

Ciliates exhibit a number of features that make it likely that a suitable DNA barcode will be identified. They are monophyletic and relatively well-studied and have a complex, character-rich morphology that allows most forms to be identified reliably to morphospecies (Corliss 2002). Despite these advantages, relatively few studies have focused on the DNA barcoding of ciliates (Barth et al. 2006; Chantangsi et al. 2007; Gentekaki and Lynn 2012; Guggiari and Peck 2008; Kher et al. 2011; Santoferrara et al. 2013; Stoeck et al. 2014; Strüder-Kypke and Lynn 2010; Zhao et al. 2013). Owing to their genetic diversity, different coding regions may be needed as barcodes for different groups of ciliates. Potential candidates for barcoding markers include the coding regions for the small subunit ribosomal DNA (SSU rDNA, 18S rDNA), large

subunit ribosomal DNA (LSU rDNA, 28S rDNA), internal transcribed spacers (ITS1 and ITS2), cytochrome b (*cytb*), and cytochrome c oxidase (*cox1*). Proofed genetic distances for members of individual major taxa *should* be established and adjusted taking into account the morphological data (Lee 2004; Lipscomb et al. 2003; Seberg et al. 2003; Will and Rubinoff 2004). Barcode data *should* be correlated with traditionally described taxa (see above) and the autecological information associated with them through names as part of the process of inferring ecological roles from occurrences documented by barcodes. Furthermore, as indicated above, voucher material showing the taxonomically relevant features *should* be deposited as a means of cross-referencing a new barcode sequence, the extracted DNA from which the barcode sequence was derived also *should* be deposited, and fixed cells and/or live or cryopreserved cultures *could* be deposited.

OTHER CHARACTERS

Behavior

There there is a history dating back to Antonie van Leeuwenhoek, of complementing descriptions of protists with ancillary information on their behavior, but the Code does not explicitly support this approach. Some ciliates, however, exhibit characteristic behavior, such as jumping several "body lengths" in a fraction of a second, spiral swimming, or adhering to surfaces. Such characters *could* be included in descriptions. It is critical to note that such behaviors are dependent on the environment (food type and abundance, temperature, pH, substrate type, light, etc.), convergent behaviors occur across taxa, and behavior can be similar at least in congeners. Behavior may thus be a poor diagnostic feature. Anecdotal observations could include: patterns of crawling or swimming (e.g. helical trajectories), jumping and escape behavior, feeding, contractility, formation of stalks, temporary attachment, alternation of rests and movements. The speed of swimming *could* be roughly estimated, e.g. using a small strip of scaled paper placed beneath a Petri dish, or knowing the width of the field of view at lower microscope magnifications. Simple trajectories plus remarks on peculiarities of movement may be sufficient, but more sophisticated methods, such as image analysis of video sequences and computer modeling of cell motion, are available (Oliveira-Pinto et al. 1993). In contrast, if a study fully characterizes the behavior of a taxon, then these data *should* be used to aid in the identification of live material and play a part in future research on functionality. We, therefore, encourage detailed studies of ciliate behavior and ecophysiology, which may then be tied to taxonomic descriptions. However, we caution against providing information as descriptive when it is not rigorously examined (with full documentation of methods and results).

Environmental data

Beyond describing the type environment, as outlined in the Code (Recommendation 76A), environmental factors

can affect behavior and have a significant impact on growth/size. Therefore, physical data such as temperature, salinity, and pH of the water, sediment, or soil from which samples are taken *should* be recorded. Likewise, detailed culture conditions *must* be presented in the methods of described species (see above).

INFORMATION RESOURCES

Overview

Both taxonomic and nontaxonomic data relating to biodiversity are often fragmentary and heterogeneous, making it difficult or impossible for researchers to identify and fill gaps in knowledge quickly and accurately. Information resources *could* reduce this issue by nourishing integrative biodiversity as an investigative approach, but data *must* be reliable and readily accessible to a broad, interdisciplinary research community. Furthermore, a good information resource *should* encourage an increasing array of links to other expert sources but not duplication of such resources. These are the aspirations we promote below.

Beyond type material, there are many objects that have value to taxonomy of ciliates (Table 1). However, at this time, no knowledge resource like GenBank or ZooBank exists for preserved cells, type slides, or other materials needed to ensure high-quality taxonomic research on ciliates. Also it is difficult to find some published material, such as taxonomic treatments in older journals that include nomenclatural acts or descriptions/revisions plus illustrations that constitute the functional equivalent of type material.

Table 1. Categories of information resources applicable to biodiversity of ciliates (modified from Baskauf 2010)

Category of resource	Form of representation	Specific representations
Physical object	Material artifact	Film negative
		Photographic print
		Film movie
		Living/viable culture
		Preserved cell/DNA sample
		Prepared slides
		Fixed specimens
		Hard-copy field notes
		Hard-copy laboratory notes
		Original illustrations
Information	Digital file	Image
		Video
		Specimen data
		DNA/RNA sequence
		Sequence alignment
		Phylogenetic tree
		Classification
		Taxonomic synonymy
		Geographic coordinates
		Physical-chemical measurements

There have been efforts to make some of these resources more available, through digitizing initiatives such as the Biodiversity Heritage Library (<http://www.biodiversitylibrary.org/>) and through taxon-specific websites such as:

- *Ciliates in Activated Sludge* (software package available for download from <http://ciliateguide.myspecies.info/ciliates-activated-sludge>)
- *Planktonic Ciliate Project* (<http://ciliate.zooplankton.cn>)
- *The Microbial Digital Specimen Archives* (Protist information Server: http://protist.i.hosei.ac.jp/pdb/image_s/menuE.html)
- *Free-Living Ciliates in the Bohai and Yellow Seas, China* (<http://www2.ouc.edu.cn/akfs/ciliate/asp/>)
- *Marine Benthic Ciliates* (<http://ciliate.myspecies.info/en>).

However, the dispersed nature of the above resources reduces the potential to interrogate them. At the very least, as a community, we *should* supplement these efforts by digitizing items that are unavailable online and/or difficult to obtain in hard copy, following these guidelines:

- Supplementary information and data linked with type or voucher specimens *should* be made available on a curated open-access website
- Sites providing access to metadata *should* be organized to allow remote interrogation (Gachon et al. 2013)
- Documentation *should* be provided so that collections of specimens comply with the Convention on Biological Diversity (<http://www.cbd.int/>)
- Standards for supplementary materials and data *should* be agreed upon by the research community
- Reliable metadata *should* be made available via a dedicated web-portal, e.g. The Global Genome Biodiversity Network [GGBN] Data Portal (<http://data.ggbn.org>) (Droege et al. 2014).

Development of resources for sharing information and identifying species

Here we suggest that integrative biodiversity of ciliates would benefit from development of a single online resource, following the criteria outlined above. There are four reasons for developing such a resource. It *should*:

- Facilitate continual, accurate identification of ciliates collected from nature
- Allow new taxonomic investigations to build on *all* prior work
- Enable taxonomic data/metadata to be combined with reliable ecological and physiological data/metadata to facilitate large-scale studies
- Provide quality control for online materials from other web sites.

To this end, we propose development of *The Ciliate Guide* (<http://ciliateguide.myspecies.info/>), which is a Scratchpad/Biodiversity online resource, providing a nomenclatural template for keys, descriptions, and metadata to the level of species. We suggest that it *should* be used as the software platform for the following reasons:

- Provides a taxonomic portal for entry of content
- Provides an ability to create keys for identification

- Provides the capacity to submit data and metadata
- Provides guaranteed permanent hosting (EU-sponsored Scratchpads project: <http://scratchpads.eu/>)
- Is freely accessible
- Can access material from independent sources
- Is suited to a communal environment that allows a registered user to contribute materials that are vetted by a select group of authoritative users.

We also suggest that the IRCN-BC *should* assume this task because of its broad, inclusive membership and focus on integrative biodiversity. Aside from providing a resource for identifying species and assembling data/metadata, *The Ciliate Guide* also can be used as a searchable database of physical specimens and type slides. Thus it can function as a "virtual museum", making it much easier to locate specimens and link metadata to digitized images of type specimens. To develop *The Ciliate Guide* the IRCN-BC **will**:

- Establish a steering group to propose the initial requirements and set priorities
- Configure *The Ciliate Guide* accordingly
- Identify complementary initiatives and establish information-flow
- Establish a registration system allowing registered users to contribute, limiting editorial rights to a committee of experts
- Promote participation to gather and review resources
- Ensure an active update of the site
- Identify and add features to accommodate developments in methodology and needs
- Establish means to provide credit for contributions.

CONCLUSIONS

Technological advances over the last several decades have made it possible to achieve rapid progress in knowledge about the biodiversity of organisms. Research on ciliates and other protists has, of course, benefited from this trend, but not to the degree seen in multicellular taxa. In essence, research on ciliate biodiversity is constrained by a deficit in basic information about their biodiversity owing to historical difficulties in making systematic observations of microorganisms. The best remedy is an organized, sustained push to follow uniform standards of practice while assembling data that will form a future foundation. To this end, we have articulated needs in the form of Grand Challenges that are addressed by sets of specific recommendations for best practice and a proposal to populate a database on the biodiversity of ciliates. A regularly updated listing of recommendations accompanied by checklists for observational methodologies will be made available on the website of the IRCN-BC (<http://ircn-bc.org>).

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