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REVIEW ARTICLE

Updating Biodiversity Studies in Loricate Protists: The Case of the Tintinnids (Alveolata, Ciliophora, Spirotrichea)

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ABSTRACT

Species determination is crucial in biodiversity research. In tintinnids, identification is based almost exclusively on the lorica, despite its frequent intraspecific variability and interspecific similarity. We suggest updated procedures for identification and, depending on the aim of the study, further steps to obtain morphological, molecular, and ecological data. Our goal is to help improving the collection of information (e.g. species re-/descriptions and DNA barcodes) that is essential for generating a natural tintinnid classification and a reliable reference for environmental surveys. These suggestions are broadly useful for protistologists because they exemplify data integration, quality/effort compromise, and the need for scientific collaborations.

ACCURATE species identification is critical to understand protist biodiversity in all its dimensions (taxonomic, genetic, and functional). It is also the only means to track species distributions and abundances, especially in the context of potential shifts due to climate change. Recent developments in sequencing technologies have led to the discovery of novel lineages, life styles, and physiological traits, and are more and more integrated in

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the protistologist's tool box. It is thus pivotal to accompany such advances in molecular methods with updated views of the classical, morphology-based taxonomies that still prevail for several protist groups, especially those with shells or other hard structures that are relatively easy to collect, preserve, examine, and classify. One of those groups are the tintinnid ciliates (Spirotrichea, Tintinnida), which are characterized by a lorica. In contrast to the vast majority of ciliates described mainly by cell morphology, the taxonomy and classification of the more than 1,000 species and 75 genera of tintinnids are based almost exclusively on lorica features (Agatha and Strüder-Kypke 2013; Lynn 2008). Also, the study of the lorica has allowed the accumulation of diversity and distribution data for more than two centuries (Alder 1999; Dolan et al. 2013), and it has even been shown to relate to ecophysiological traits (Dolan 2010).

Despite the invaluable knowledge generated using lorica-based species identification, the power of this structure for taxa circumscription and genealogical reconstruction has long been questioned (Entz 1909), but rarely confirmed due to the paucity in the application of other criteria. Examples of phenotypic plasticity observed in cultures (Laval-Peuto 1981) and of cryptic species differentiated by their DNA sequences (Santoferrara et al. 2013, 2015) have shown that lorica taxonomy is partly artificial and that the actual tintinnid diversity is unknown.

Data on cell morphology, lorica ultrastructure, and DNA sequences have recently allowed some taxonomic rearrangements. For example, *Favella ehrenbergii*, an important model organism in plankton ecology, has actually included species from two different families (Agatha and Strüder-Kypke 2012). But a far-reaching revision of tintinnid systematics is currently impossible because cytological and molecular characters are known in less than 10% of the named species (Agatha and Strüder-Kypke 2014). Hence, the lorica is still the only key to the comprehensive, up to 240-yr-old body of literature, including almost all original descriptions.

We present an updated view on the use of the lorica for species identification and subsequent steps for taxonomic and ecological work (Fig. 1). We gathered the morphological, molecular, and ecological data that, according to our experience, provide the best compromise between data quality and work effort based on the desired aim of the study (Boxes S1–S3). Integrated approaches are needed to avoid old and recent concerns, such as the erection of insufficiently described taxa and the accumulation of inaccurately identified DNA sequences in public repositories (e.g. NCBI GenBank). These problems affect all aspects of biodiversity, from the establishment of a natural classification system and the elucidation of phylogenetic relationships to the exploration of community structure and function using environmental sequencing. We hope to improve data quality by guiding new generations of specialists and encouraging collaborations among researchers with different expertises.

SPECIES IDENTIFICATION

Identification is based on lorica morphology (Box S1.1). The general lorica shape, the ability to adhere particles onto some parts or the entire lorica, and the diameter and characteristics of the lorica opening are the most reliable taxonomic characters (Laval-Peuto and Brownlee 1986). In contrast, the length and other lorica features are more variable, as they depend on the construction stage and are influenced by the cell cycle and environmental factors.

Documentation of lorica morphology and morphometry, as well as of the literature used for determination (including the discussion of observed deviations), is necessary for future comparisons. Monographs and revisionary taxonomic treatises are a very helpful starting point for identification (e.g. Kofoid and Campbell 1929, 1939; Zhang et al. 2012), but these works usually changed the original species circumscriptions (by "splitting" or "lumping" them artifactually), occasionally do not provide the relevant information in a suitable way, and sometimes even include mistakes. To overcome these difficulties, it is better to "go back to the roots" and rescue the old bibliography (e.g. Brandt 1906, 1907; Hada 1932; Jörgensen 1924). The usage of original descriptions or, under justified circumstances, authoritative redescriptions (those that allow an unequivocal identification in cases of insufficient original descriptions) helps to prevent mistakes in the final identifications. Consulting original descriptions is now feasible given that many of the older publications are freely available online (https://archive.org; http://www.biodiversitylibrary.org; http://www.ioc-unesco.org; http://www.obs-vlfr.fr/LOV/aquaparadox/html/ClassicMonographs.php).

OTHER ASPECTS INVOLVED IN UNDERSTANDING TINTINNID BIODIVERSITY

Species abundance and distribution

The classical approach of lorica-based identification (Box S1.1) combined with counts and measurements under the inverted microscope (Box S3) is currently the most accurate and simplest way to estimate abundance and biomass. This method is still widely used to study distribution over spatial and temporal scales (McManus and Santoferrara 2013).

More recently, the use of environmental sequencing (clone libraries and, lately, high-throughput sequencing, HTS) to study the diversity and distribution of tintinnid assemblages has become promising, for example, for the detection of rare or cryptic taxa not observed by microscopy (Bachy et al. 2013, 2014; Santoferrara et al. 2014, 2016). These methods generally use partial sequences of the small subunit ribosomal RNA gene (SSU rDNA) and involve several bioinformatic procedures (Bik et al. 2012; Logares et al. 2012). Sequences are clustered into operational taxonomic units (OTUs) based on their similarity (generally 99–100% for tintinnids). These OTUs may be identified using reference data-

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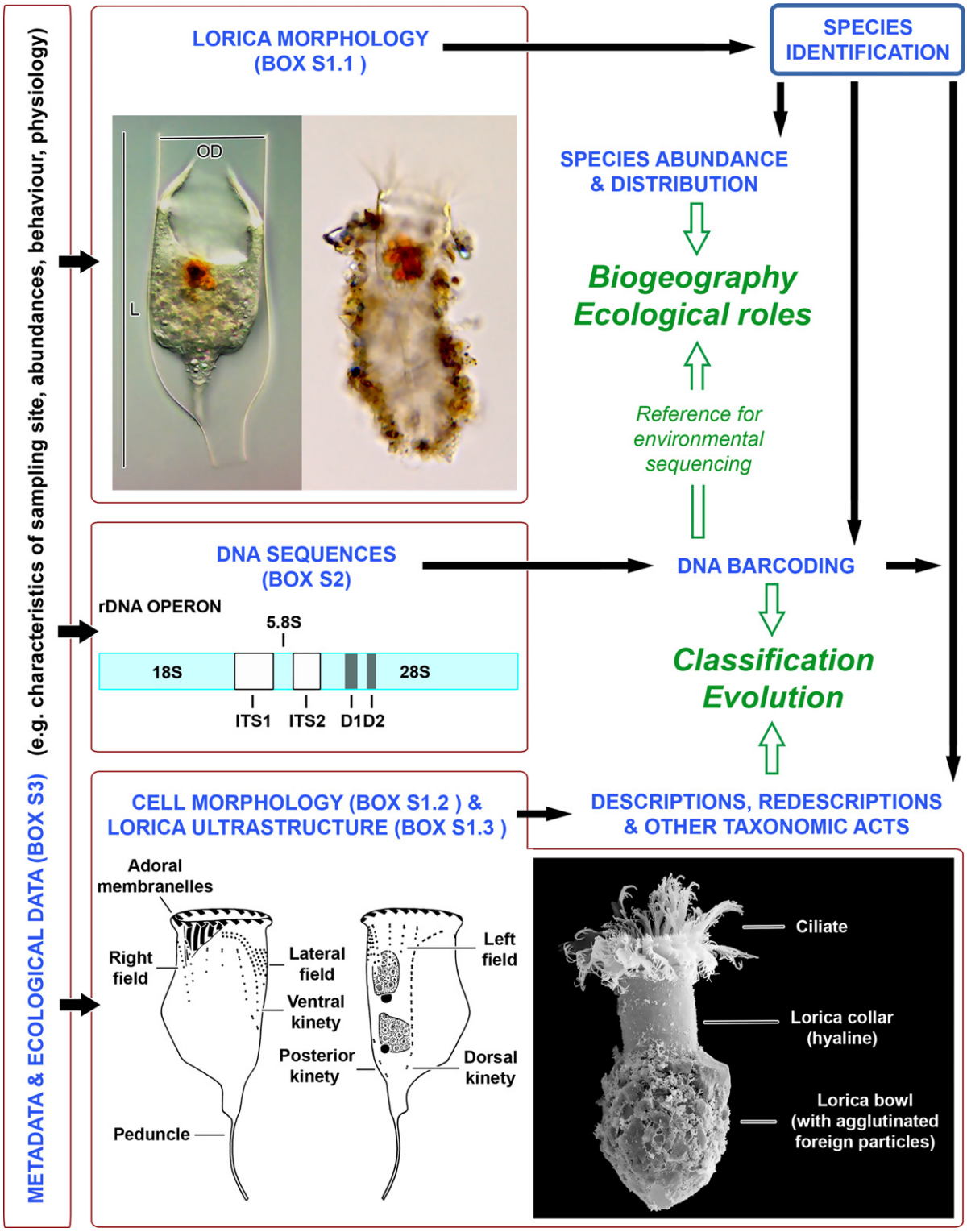

Figure 1 Biodiversity studies in tintinnid ciliates. Species identification is still based almost exclusively on lorica features, such as the structure, shape, and size, especially of the opening diameter. Accurate determination is fundamental for subsequent steps, such as (i) estimation of species abundance and distribution for ecological studies, (ii) linkage of DNA sequences to species for phylogenetic inference and the establishment of reference databases, and (iii) the re-/description of species based not only on lorica characters, but also on cell features recognizable in live and protargol-stained material. Integration of morphological, molecular, and ecological information (Boxes S1–S3) is needed to provide a natural classification system, a reliable evolutionary model, and a deep understating of biogeography and ecological roles. L, length; OD, opening diameter.

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bases (see below), given the usual desire to link OTUs to traditional species in terms of morphology, behaviour, ecology and/or physiology (Caron 2013; Dolan 2015; McManus and Katz 2009). A limitation of environmental sequencing, however, is that it provides only relative abundances, which sometimes are inconsistent compared to cell counts (for example, due to biases during DNA extraction and PCR amplification or because of differences in the number of SSU rDNA gene copies among species; Medinger et al. 2010).

DNA barcoding

Barcoding involves two stages. Its ultimate aim is to identify query DNA sequences (e.g. environmental OTUs) using reference DNA sequences. But first, reference DNA sequences from identified species have to be obtained. This stage requires accuracy in both species identification (Box S1.1) and molecular analyses (Box S2). For tintinnids, the sequencing of isolated single cells offers a direct link between lorica morphology and DNA sequence, and it is useful in field samples or species not amenable to clonal culture (Lynn and Pinheiro 2009). Given that single cell sequencing results in specimen destruction, published measurements and images are the accompanying evidence for the barcoded species (Pawlowski et al. 2012).

The SSU rDNA gene is the most commonly sequenced marker in tintinnids and has been very useful for phylogenetic inferences at family and genus levels (Agatha and Strüder-Kypke 2014). However, the differentiation of closely related species is better achieved by less conserved regions of the rDNA, such as the D1–D2 region of the large subunit rRNA gene (LSU rDNA) and the 5.8S rRNA gene combined with the internally transcribed spacers (ITS) 1 and 2 (Santoferrara et al. 2013, 2015; Xu et al. 2012). Most tintinnid species sequenced so far differ by at least 0.6% in the LSU rDNA and/or 1.5% in the ITS regions (Santoferrara et al. 2015). Analysing all the rDNA regions simultaneously improves phylogeny resolution and allows focusing on different systematic levels (species, genus, and above-genus) by creating sub-datasets of conserved and hypervariable regions (Bachy et al. 2012; Santoferrara et al. 2012). In contrast, the use of the ITS2 secondary structure and compensatory base changes for species separation is controversial and should be cautiously evaluated (Caisova et al. 2011; Coleman 2000). The proposed universal metazoan barcode, the mitochondrial cytochrome oxidase subunit I gene, has not been reliably amplified for tintinnids yet (Strüder-Kypke and Lynn 2010).

Building a reference database for molecular identification and phylogeny requires careful scrutiny of DNA sequences retrieved from public repositories (e.g. NCBI GenBank) given the proliferation of errors and misidentifications. An alternative starting point are public reference databases, in which the DNA sequences are (at least partially) curated based on taxonomic expertise (e.g. PR^2, Guillou et al. 2013; EukRef, http://eukref.org/).

Descriptions, redescriptions, and nomenclatural changes

Taxonomic acts are regulated by the International Code of Zoological Nomenclature (ICZN = International Commission of Zoological Nomenclature 1999). For the description of new species and the redescription of insufficiently known species of tintinnids, it is indispensable that the lorica information necessary for identification is complemented by additional lorica and cytological features as detailed as possible (Boxes S1.2 and S1.3). Permanent material must be deposited in an acknowledged collection, and at least SSU rDNA sequences should be submitted to public repositories (e.g. Lynn and Simpson 2009).

A combination of live observation and protargol staining reveals the majority of species-specific morphological features in most ciliate groups, and the latter provides permanent slides for deposition (Foissner 2014). Low numbers and/or poorly stained cells often prevent accurate descriptions and redescriptions. It is difficult to predetermine a number of specimens to study, yet morphometric data and statistical analyses should be grounded on enough specimens to grant the best compromise between effort and accuracy. An adequate sample size avoids the establishment of new species based on possibly atypical specimens and allows rough estimates of the intraspecific variability; in this sense, it is also important to study several populations.

Species under scrutiny need careful comparison with congeners and other similar species, including the discussion of resemblances and differences in morphological and molecular characters. Currently, the scarce knowledge on intraspecific and interspecific variability in cell morphology (only about 30 species have been studied cytologically, generally based on up to 30 individuals from single populations; Agatha and Strüder-Kypke 2013) and the lack of an absolute barcode gap in the commonly used molecular markers (Santoferrara et al. 2015) hamper unequivocal separation or lumping of species. Therefore, we encourage comparing as many features as possible (e.g. multiple morphological and molecular parameters, biogeography, ecophysiology), which also maximises the chance of identifying new diagnostic characters. Synonymizations and other nomenclatural changes should never be grounded on weak evidence (e.g. gene phylogenies of species potentially misidentified by their lorica). Taxa reclassifications should only be performed when the diagnoses can be improved by reliable morphological and/or genetic synapomorphies from accurately identified specimens.

CONCLUSIONS

The "taxonomic impediment," that is, the decline in taxonomic knowledge together with the number of classical taxonomists, limits the accuracy of species identification and the adequacy of species descriptions and redescriptions. At first glance, it seems desirable to accelerate the pace of species investigations by adopting a "turbo-taxonomy" approach, focussing on a few, easily accessible morphologi-

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cal features, which is particularly tempting using the tintinnid lorica. However, in the long run, it might turn out that these rapidly produced data (e.g. during species discovery or DNA barcoding) lack relevant information or cause errors, thereby increasing confusion in taxonomic and ecological work.

Future directions in tintinnid studies should include the integral redescription of type species, which may be difficult to sample even in the type localities, but would enable taxonomic rearrangements needed in several families and genera (Agatha and Strüder-Kypke 2014). Other species of particular interest are the ones from comparatively less studied environments, such as the open ocean. These species are challenging as their low abundances hamper the collection of sufficient material and some of their features are difficult to study during oceanographic expeditions. But even the insufficiently known species that are abundant in coastal waters are worth examination, because they might possess surprising morphological features and can provide useful DNA barcodes. This information is crucial to build accurate, public databases of reference sequences, especially now that tintinnids have started to be tackled using HTS and "-omics" approaches (Bachy et al. 2013; Keeling et al. 2014).

Complementary morphological, molecular, and ecological data are needed to provide key insights, namely, a natural classification system, a reliable evolutionary model, and a deep understating of biogeography and ecological roles (Fig. 1). To facilitate this aim, we provide checklists and recommendations for data collection and evaluation (Boxes S1–S3). The collaboration of experts in different disciplines may be the most productive way to carry out such integrated biodiversity studies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Box S1. Morphological data.
Box S2. Molecular data.
Box S3. Metadata and ecological data.

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