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

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Running head: Biodiversity studies in tintinnid ciliates

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47 **ABSTRACT**
48 Species determination is crucial in biodiversity research. In tintinnids, identification is based
49 almost exclusively on the lorica, despite its frequent intraspecific variability and interspecific
50 similarity. We suggest updated procedures for identification and, depending on the aim of the
51 study, further steps to obtain morphological, molecular, and ecological data. Our goal is to help
52 improving the collection of information (e.g., species re-/descriptions and DNA barcodes) that is
53 essential for generating a natural tintinnid classification and a reliable reference for
54 environmental surveys. These suggestions are broadly useful for protistologists because they
55 exemplify data integration, quality/effort compromise, and the need for scientific collaborations.
56
57 **Keyword**s
58 Biodiversity; DNA barcoding; ecology; morphology; phylogeny; taxonomy.
59
60
61 Accurate species identification is critical to understand protist biodiversity in all its dimensions
62 (taxonomic, phylogenetic, and functional). It is also the only means to track species distributions
63 and abundances, especially in the context of potential shifts due to climate change. Recent
64 developments in sequencing technologies have led to the discovery of novel lineages, life styles,
65 and physiological traits, and are more and more integrated in the protistologist's tool box. It is
66 thus pivotal to accompany such advances in molecular methods with updated views of the
67 classical, morphology-based taxonomies that still prevail for several protist groups, especially
68 those with shells or other hard structures that are relatively easy to collect, preserve, examine,
69 and classify. One of those groups are the tintinnid ciliates (Spirotrichea, Tintinnida), which are
70 characterized by a lorica. In contrast to the vast majority of ciliates (described mainly by cell
71 morphology), the taxonomy and classification of the more than one thousand species and
72 seventy-five genera of tintinnids are based almost exclusively on lorica features (Agatha and
73 Strüder-Kypke 2013; Lynn 2008). Also, the study of the lorica has allowed the accumulation of
74 diversity and distribution data for more than two centuries (Alder 1999; Dolan et al. 2013), and it
75 has even been shown to relate to ecophysiological traits (Dolan 2010).
76 Despite the invaluable knowledge generated using lorica-based species identification,
77 the power of this structure for taxa circumscription and genealogical reconstruction has long
78 been questioned (Entz 1909), but rarely confirmed due to the paucity in the application of other
79 criteria. Examples of phenotypic plasticity observed in cultures (Laval-Peuto 1981) and of
80 cryptic species differentiated by their DNA sequences (Santoferrara et al. 2013, 2015) have
81 shown that lorica taxonomy is partly artificial and that the actual tintinnid diversity is unknown.
82 Data on cell morphology, lorica ultrastructure, and DNA sequences have recently
83 allowed some taxonomic rearrangements. For example, *Favella ehrenbergii*, an important model
84 organism in plankton ecology, has actually included species from two different families (Agatha
85 and Strüder-Kypke 2012). But a far-reaching revision of tintinnid systematics is currently
86 impossible because cytological and molecular characters are known in less than 10% of the
87 named species (Agatha and Strüder-Kypke 2014). Hence, the lorica is still the only key to the
88 comprehensive, up to 240-year-old body of literature, including almost all original descriptions.
89 We present an updated view on the use of the lorica for species identification and
90 subsequent steps for taxonomic and ecological work (Fig. 1). We gathered the morphological,
91 molecular, and ecological data that, according to our experience, provide the best compromise
92 between data quality and work effort depending on the desired aim of the study (Supporting

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93 Information: Box S1, S2, S3). Integrated approaches are needed to avoid old and recent
94 concerns, such as the erection of insufficiently described taxa and the accumulation of
95 inaccurately identified DNA sequences in public repositories (e.g., NCBI GenBank). These
96 problems affect all aspects of biodiversity, from the establishment of a natural classification
97 system and the elucidation of phylogenetic relationships to the exploration of community
98 structure and function using environmental sequencing. We hope to improve data quality by
99 guiding new generations of specialists and suggesting collaborations among researchers with
100 different expertises.
101
102

103 **SPECIES IDENTIFICATION**

104 Identification is based on lorica morphology (Box S1.1). The general lorica shape, the ability to
105 adhere particles onto some parts or the entire lorica, and the diameter and characteristics of the
106 lorica opening are the most reliable taxonomic characters (Laval-Peuto and Brownlee 1986). In
107 contrast, the length and other lorica features are more variable, as they depend on the
108 construction stage and are influenced by the cell cycle and environmental factors.
109 Documentation of lorica morphology and morphometry as well as of the literature used
110 for determination (including the discussion of observed deviations) is necessary for future
111 comparisons. Monographs and revisionary taxonomic treatises are a very helpful starting point
112 for identification (e.g., Kofoid and Campbell 1929, 1939; Zhang et al. 2012), but these works
113 usually changed the original species circumscriptions (by "splitting" or "lumping" them
114 artifactually), occasionally do not provide the relevant information in a suitable way, and
115 sometimes even include mistakes. To overcome these difficulties, it is better to "go back to the
116 roots" and rescue the information from old bibliography (e.g., Brandt 1906, 1907; Hada 1932;
117 Jörgensen 1924). The usage of original descriptions or, under justified circumstances,
118 authoritative redescriptions (those that allow an unequivocal identification in cases of
119 insufficient original descriptions) helps to prevent mistakes in the final identifications.
120 Consulting original descriptions is now feasible given that many of the older publications are
121 freely available online (https://archive.org; http://www.biodiversitylibrary.org; http://www.ioc-
122 unesco.org; http://www.obs-vlfr.fr/LOV/aquaparadox/html/ClassicMonographs.php).
123
124

125 **OTHER ASPECTS INVOLVED IN UNDERSTANDING TINTINNID BIODIVERSITY**

126 **Species abundance and distribution**

127 The classical approach of lorica-based identification (Box S1.1) combined with counts and
128 measurements under the inverted microscope (Box S3) is the most accurate and simplest way to
129 estimate abundance and biomass. This method is still widely used to study distribution over
130 spatial and temporal scales (McManus and Santoferrara 2013).
131 More recently, the use of environmental sequencing (clone libraries and, lately, high-
132 throughput sequencing, HTS) to study the diversity and distribution of tintinnid assemblages has
133 become promising, for example, for the detection of rare or cryptic taxa not observed by
134 microscopy (Bachy et al. 2013, 2014; Santoferrara et al. 2014). These methods generally use
135 partial sequences of the small subunit ribosomal RNA gene (SSU rDNA) and involve several
136 bioinformatic procedures (Bik et al. 2012; Logares et al. 2012). Sequences are clustered into
137 operational taxonomic units (OTUs) based on their similarity (generally 99-100% for tintinnids).
138 These OTUs may be identified using reference databases (see below), given the usual desire to

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139 link OTUs to traditional species in terms of morphology, behaviour, ecology and/or physiology
140 (Caron 2013; Dolan 2015; McManus and Katz 2009). A limitation of environmental sequencing,
141 however, is that it provides only relative abundances, which sometimes are inconsistent
142 compared to cell counts (for example, due to biases during DNA extraction and PCR
143 amplification or because of differences in the number of SSU rDNA gene copies among species;
144 Medinger et al. 2010).
145
146 **DNA barcoding**
147 Barcoding involves two stages. Its ultimate aim is to identify query DNA sequences (e.g.,
148 environmental OTUs) using reference DNA sequences. But first, reference DNA sequences from
149 identified species have to be obtained. This stage requires accuracy in both species identification
150 (Box S1.1) and molecular analyses (Box S2). For tintinnids, the sequencing of isolated single
151 cells offers a direct link between lorica morphology and DNA sequence, and it is useful in field
152 samples or species not amenable to clonal culture (Lynn and Pinheiro 2009). Given that single
153 cell sequencing results in specimen destruction, published measurements and images are the
154 accompanying evidence for the barcoded species (Pawlowski et al. 2012).
155 The SSU rDNA gene is the most commonly sequenced marker in tintinnids and has
156 been very useful for phylogenetic inferences at family and genus levels (Agatha and Strüder-
157 Kypke 2014). However, the differentiation of closely related species is better achieved by less
158 conserved regions of the rDNA, such as the D1-D2 region of the large subunit rRNA gene (LSU
159 rDNA) and the 5.8S rRNA gene combined with the internally transcribed spacers (ITS) 1 and 2
160 (Santoferrara et al. 2013, 2015; Xu et al. 2012). Most tintinnid species sequenced so far differ by
161 at least 0.6% in the LSU rDNA and/ or 1.5% in the ITS regions (Santoferrara et al. 2015).
162 Analysing all the rDNA regions simultaneously improves phylogeny resolution and allows
163 focusing on different systematic levels (species, genus, and above-genus rank) by creating sub-
164 databases of conserved and hypervariable regions (Bachy et al. 2012; Santoferrara et al. 2012).
165 In contrast, the use of the ITS2 secondary structure and compensatory base changes for species
166 separation is controversial and should be cautiously evaluated (Caisova et al. 2011; Coleman
167 2000). The proposed universal metazoan barcode, the mitochondrial cytochrome oxidase subunit
168 I gene, has not been reliably amplified for tintinnids yet (Strüder-Kypke and Lynn 2010).
169 Building a reference database for molecular identification and phylogeny requires
170 careful scrutiny of DNA sequences retrieved from public repositories (e.g., NCBI GenBank)
171 given the proliferation of errors and misidentifications. An alternative starting point are public
172 reference databases, in which the DNA sequences are (at least partially) curated based on
173 taxonomic and phylogenetic expertise (e.g., PR^2, Guillou et al. 2013; EukRef, http://eukref.org/).
174
175 **Descriptions, redescriptions, and nomenclature changes**
176 Taxonomic acts are regulated by the International Code of Zoological Nomenclature (ICZN
177 1999). For the description of new species and the redescription of insufficiently known species
178 of tintinnids, it is indispensable that the lorica information necessary for identification is
179 complemented by additional lorica and cytological features as detailed as possible (Box S1.2,
180 S1.3). Permanent material must be deposited in an acknowledged collection (ICZN 1999), and at
181 least SSU rDNA sequences should be submitted to public repositories (Lynn and Simpson 2009).
182 A combination of live observation and protargol staining reveals the majority of
183 species-specific morphological features in most ciliate groups and the latter provides permanent
184 slides for deposition (Foissner 2014). Low numbers and/or poorly stained cells often prevent

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185 accurate descriptions and redescriptions. It is difficult to predetermine a number of specimens to
186 study, yet morphometric data and statistical analyses should be grounded on enough specimens
187 to grant the best compromise between effort and accuracy. An adequate sample size avoids the
188 establishment of new species based on possibly atypical specimens and allows rough estimates
189 of the intraspecific variability; in this sense, it is also important to study several populations.
190 Species under scrutiny need careful comparison with congeners and other similar
191 species, including the discussion of resemblances and differences in morphological and
192 molecular characters. Currently, the scarce knowledge on intraspecific and interspecific
193 variability in cell morphology (only about 30 species have been studied cytologically, generally
194 based on up to 30 individuals from single populations; Agatha and Strüder-Kypke 2013) as well
195 as the lack of an absolute barcode gap in the commonly used molecular markers (Santoferrara et
196 al. 2015) makes difficult separating or lumping species unequivocally. Therefore, we encourage
197 comparing as many features as possible (e.g., multiple morphological and molecular parameters,
198 biogeography, ecophysiology), which also maximises the chance of identifying new diagnostic
199 characters. Synonymizations and other nomenclature changes should never be grounded on weak
200 evidence (e.g., gene phylogenies of species potentially misidentified by their lorica). Taxa
201 reclassifications should only be performed when the diagnosis can be improved by reliable
202 morphological and/or genetic synapomorphies from accurately identified specimens.
203
204

205 **CONCLUSIONS**

206 The 'taxonomic impediment', i.e., the decline in taxonomic knowledge together with the number
207 of classical taxonomists, limits the accuracy of species identification and the adequacy of species
208 descriptions and redescriptions (Wägele et al. 2011). At first glance, it seems desirable to
209 accelerate the pace of species investigations by adopting a "turbo-taxonomy" approach,
210 focussing on a few, easily accessible morphological features, which is particularly tempting
211 using the tintinnid lorica. However, in the long run, it might turn out that these rapidly produced
212 data (e.g., during species discovery or DNA barcoding) lack relevant information or cause errors,
213 thereby increasing confusion in taxonomic and ecological work.
214 Future directions in tintinnid studies should include the integral redescription of type
215 species, which are difficult to sample even in the type localities, but would enable taxonomic
216 rearrangements needed in several families and genera (Agatha and Strüder-Kypke 2014). Other
217 species of interest are the ones from comparatively less studied environments, such as the open
218 ocean, which are more challenging as their low abundances hamper the collection of sufficient
219 material and some of their features are more difficult to study during oceanographic expeditions.
220 But even the insufficiently known species that are abundant in coastal waters are worth
221 examination, because they might possess surprising morphological features and can provide
222 useful DNA barcodes. This information is crucial to build accurate, public databases of reference
223 sequences, especially in the context of current advancements that are allowing to tackle tintinnids
224 from an "-omics" perspective (Bachy et al. 2013; Keeling et al. 2014).
225 Complementary morphological, molecular, and ecological data are needed to provide
226 key insights, namely, a natural classification system, a reliable evolutionary model, and a deep
227 understating of biogeography and ecological roles (Fig. 1). To facilitate this aim, we provide
228 checklists and recommendations for data collection and evaluation (Box S1, S2, S3). The
229 collaboration of experts in different disciplines may be the most productive way to carry out such
230 integrated biodiversity studies.

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238
239
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374 **FIGURE LEGEND**
375 **Figure 1.** Biodiversity studies in tintinnid ciliates. Species identification is still based almost
376 exclusively on lorica features such as the structure, shape, and size, especially of the oral
377 diameter (OD). Accurate determination is fundamental for subsequent steps such as (i)
378 estimation of species abundance and distribution for ecological studies, (ii) linkage of DNA
379 sequences to species for phylogenetic inference and the establishment of reference databases,
380 and (iii) the re-/description of species based not only on lorica characters, but also on cell
381 features recognizable in live and protargol stained material. Integration of morphological,
382 molecular, and ecological information (Supporting Information: Box S1, S2, S3) is needed to
383 provide a natural classification system, a reliable evolutionary model, and a deep understating of
384 biogeography and ecological roles. L, length.
385
386
387 **SUPPORTING INFORMATION**
388 Box S1. Morphological data
389 Box S2. Molecular data
390 Box S3. Metadata and ecological data

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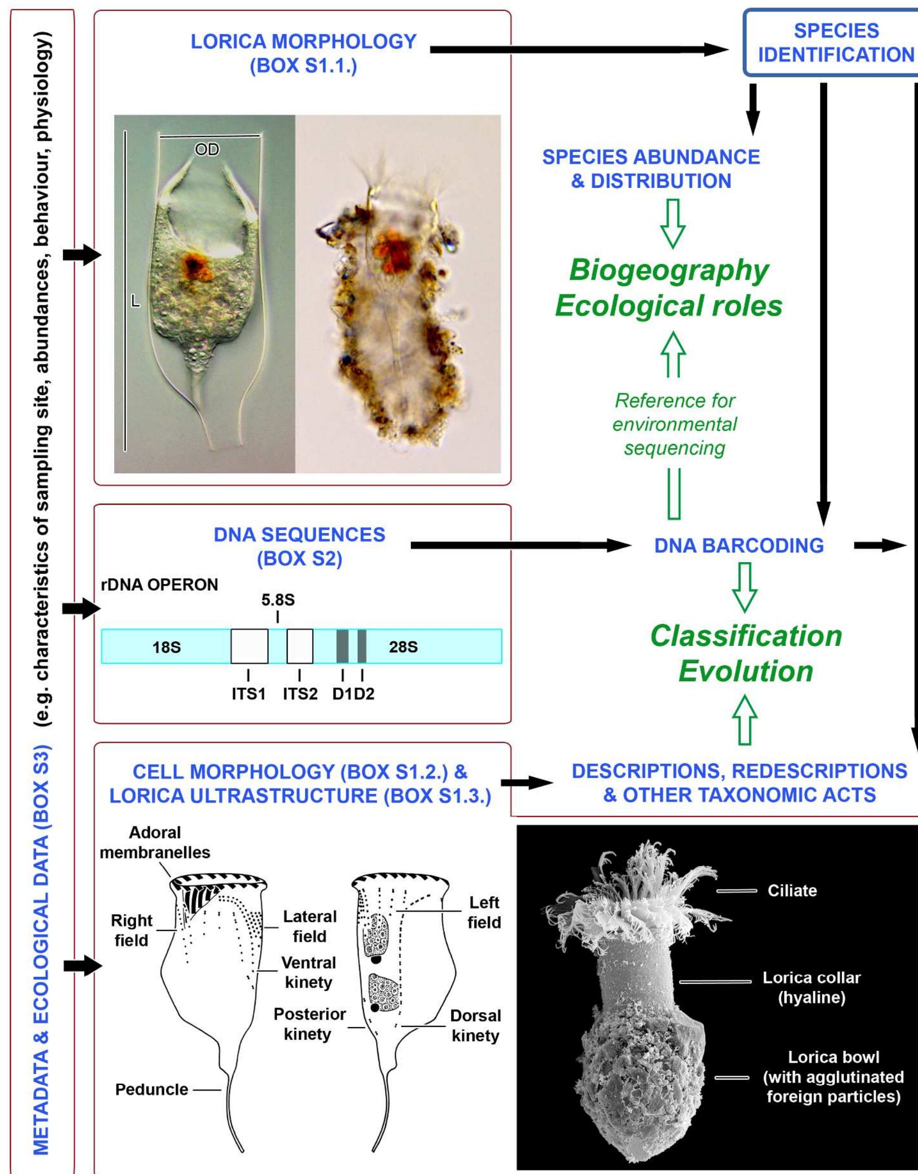

Figure 1
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Updating Biodiversity Studies in Loricate Protists: the Case of the Tintinnids (Alveolata, Ciliophora, Spirotrichea)

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Running head: Biodiversity studies in tintinnid ciliates

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47 **ABSTRACT**
48 Species determination is crucial in biodiversity research. In tintinnids, identification is based
49 almost exclusively on the lorica, despite its frequent intraspecific variability and interspecific
50 similarity. We suggest updated procedures for identification and, depending on the aim of the
51 study, further steps to obtain morphological, molecular, and ecological data. Our goal is to help
52 improving the collection of information (e.g., species re-/descriptions and DNA barcodes) that is
53 essential for generating a natural tintinnid classification and a reliable reference for
54 environmental surveys. These suggestions are broadly useful for protistologists because they
55 exemplify data integration, quality/effort compromise, and the need for scientific collaborations.
56
57 **Keywords**
58 Biodiversity; DNA barcoding; ecology; morphology; phylogeny; taxonomy.
59
60
61 Accurate species identification is critical to understand protist biodiversity in all its dimensions
62 (taxonomic, phylogenetic, and functional). It is also the only means to track species distributions
63 and abundances, especially in the context of potential shifts due to climate change. Recent
64 developments in sequencing technologies have led to the discovery of novel lineages, life styles,
65 and physiological traits, and are more and more integrated in the protistologist's tool box. It is
66 thus pivotal to accompany such advances in molecular methods with updated views of the
67 classical, morphology-based taxonomies that still prevail for several protist groups, especially
68 those with shells or other hard structures that are relatively easy to collect, preserve, examine,
69 and classify. One of those groups are the tintinnid ciliates (Spirotrichea, Tintinnida), which are
70 characterized by a lorica. In contrast to the vast majority of ciliates (described mainly by cell
71 morphology), the taxonomy and classification of the more than one thousand species and
72 seventy-five genera of tintinnids are based almost exclusively on lorica features (Agatha and
73 Strüder-Kypke 2013; Lynn 2008). Also, the study of the lorica has allowed the accumulation of
74 diversity and distribution data for more than two centuries (Alder 1999; Dolan et al. 2013), and it
75 has even been shown to relate to ecophysiological traits (Dolan 2010).
76 Despite the invaluable knowledge generated using lorica-based species identification,
77 the power of this structure for taxa circumscription and genealogical reconstruction has long
78 been questioned (Entz 1909), but rarely confirmed due to the paucity in the application of other
79 criteria. Examples of phenotypic plasticity observed in cultures (Laval-Peuto 1981) and of
80 cryptic species differentiated by their DNA sequences (Santoferrara et al. 2013, 2015) have
81 shown that lorica taxonomy is partly artificial and that the actual tintinnid diversity is unknown.
82 Data on cell morphology, lorica ultrastructure, and DNA sequences have recently
83 allowed some taxonomic rearrangements. For example, *Favella ehrenbergii*, an important model
84 organism in plankton ecology, has actually included species from two different families (Agatha
85 and Strüder-Kypke 2012). But a far-reaching revision of tintinnid systematics is currently
86 impossible because cytological and molecular characters are known in less than 10% of the
87 named species (Agatha and Strüder-Kypke 2014). Hence, the lorica is still the only key to the
88 comprehensive, up to 240-year-old body of literature, including almost all original descriptions.
89 We present an updated view on the use of the lorica for species identification and
90 subsequent steps for taxonomic and ecological work (Fig. 1). We gathered the morphological,
91 molecular, and ecological data that, according to our experience, provide the best compromise
92 between data quality and work effort depending on the desired aim of the study (Supporting

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93 Information: Box S1, S2, S3). Integrated approaches are needed to avoid old and recent
94 concerns, such as the erection of insufficiently described taxa and the accumulation of
95 inaccurately identified DNA sequences in public repositories (e.g., NCBI GenBank). These
96 problems affect all aspects of biodiversity, from the establishment of a natural classification
97 system and the elucidation of phylogenetic relationships to the exploration of community
98 structure and function using environmental sequencing. We hope to improve data quality by
99 guiding new generations of specialists and suggesting collaborations among researchers with
100 different expertises.
101
102
103 **SPECIES IDENTIFICATION**
104 Identification is based on lorica morphology (Box S1.1). The general lorica shape, the ability to
105 adhere particles onto some parts or the entire lorica, and the diameter and characteristics of the
106 lorica opening are the most reliable taxonomic characters (Laval-Peuto and Brownlee 1986). In
107 contrast, the length and other lorica features are more variable, as they depend on the
108 construction stage and are influenced by the cell cycle and environmental factors.
109 Documentation of lorica morphology and morphometry as well as of the literature used
110 for determination (including the discussion of observed deviations) is necessary for future
111 comparisons. Monographs and revisionary taxonomic treatises are a very helpful starting point
112 for identification (e.g., Kofoid and Campbell 1929, 1939; Zhang et al. 2012), but these works
113 usually changed the original species circumscriptions (by "splitting" or "lumping" them
114 artifactually), occasionally do not provide the relevant information in a suitable way, and
115 sometimes even include mistakes. To overcome these difficulties, it is better to "go back to the
116 roots" and rescue the information from old bibliography (e.g., Brandt 1906, 1907; Hada 1932;
117 Jörgensen 1924). The usage of original descriptions or, under justified circumstances,
118 authoritative redescriptions (those that allow an unequivocal identification in cases of
119 insufficient original descriptions) helps to prevent mistakes in the final identifications.
120 Consulting original descriptions is now feasible given that many of the older publications are
121 freely available online (https://archive.org; http://www.biodiversitylibrary.org; http://www.ioc-
122 unesco.org; http://www.obs-vlfr.fr/LOV/aquaparadox/html/ClassicMonographs.php).
123
124
125 **OTHER ASPECTS INVOLVED IN UNDERSTANDING TINTINNID BIODIVERSITY**
126 **Species abundance and distribution**
127 The classical approach of lorica-based identification (Box S1.1) combined with counts and
128 measurements under the inverted microscope (Box S3) is the most accurate and simplest way to
129 estimate abundance and biomass. This method is still widely used to study distribution over
130 spatial and temporal scales (McManus and Santoferrara 2013).
131 More recently, the use of environmental sequencing (clone libraries and, lately, high-
132 throughput sequencing, HTS) to study the diversity and distribution of tintinnid assemblages has
133 become promising, for example, for the detection of rare or cryptic taxa not observed by
134 microscopy (Bachy et al. 2013, 2014; Santoferrara et al. 2014). These methods generally use
135 partial sequences of the small subunit ribosomal RNA gene (SSU rDNA) and involve several
136 bioinformatic procedures (Bik et al. 2012; Logares et al. 2012). Sequences are clustered into
137 operational taxonomic units (OTUs) based on their similarity (generally 99-100% for tintinnids).
138 These OTUs may be identified using reference databases (see below), given the usual desire to

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139 link OTUs to traditional species in terms of morphology, behaviour, ecology and/or physiology
140 (Caron 2013; Dolan 2015; McManus and Katz 2009). A limitation of environmental sequencing,
141 however, is that it provides only relative abundances, which sometimes are inconsistent
142 compared to cell counts (for example, due to biases during DNA extraction and PCR
143 amplification or because of differences in the number of SSU rDNA gene copies among species;
144 Medinger et al. 2010).
145
146 **DNA barcoding**
147 Barcoding involves two stages. Its ultimate aim is to identify query DNA sequences (e.g.,
148 environmental OTUs) using reference DNA sequences. But first, reference DNA sequences from
149 identified species have to be obtained. This stage requires accuracy in both species identification
150 (Box S1.1) and molecular analyses (Box S2). For tintinnids, the sequencing of isolated single
151 cells offers a direct link between lorica morphology and DNA sequence, and it is useful in field
152 samples or species not amenable to clonal culture (Lynn and Pinheiro 2009). Given that single
153 cell sequencing results in specimen destruction, published measurements and images are the
154 accompanying evidence for the barcoded species (Pawlowski et al. 2012).
155 The SSU rDNA gene is the most commonly sequenced marker in tintinnids and has
156 been very useful for phylogenetic inferences at family and genus levels (Agatha and Strüder-
157 Kypke 2014). However, the differentiation of closely related species is better achieved by less
158 conserved regions of the rDNA, such as the D1-D2 region of the large subunit rRNA gene (LSU
159 rDNA) and the 5.8S rRNA gene combined with the internally transcribed spacers (ITS) 1 and 2
160 (Santoferrara et al. 2013, 2015; Xu et al. 2012). Most tintinnid species sequenced so far differ by
161 at least 0.6% in the LSU rDNA and/ or 1.5% in the ITS regions (Santoferrara et al. 2015).
162 Analysing all the rDNA regions simultaneously improves phylogeny resolution and allows
163 focusing on different systematic levels (species, genus, and above-genus rank) by creating sub-
164 databases of conserved and hypervariable regions (Bachy et al. 2012; Santoferrara et al. 2012).
165 In contrast, the use of the ITS2 secondary structure and compensatory base changes for species
166 separation is controversial and should be cautiously evaluated (Caisova et al. 2011; Coleman
167 2000). The proposed universal metazoan barcode, the mitochondrial cytochrome oxidase subunit
168 I gene, has not been reliably amplified for tintinnids yet (Strüder-Kypke and Lynn 2010).
169 Building a reference database for molecular identification and phylogeny requires
170 careful scrutiny of DNA sequences retrieved from public repositories (e.g., NCBI GenBank)
171 given the proliferation of errors and misidentifications. An alternative starting point are public
172 reference databases, in which the DNA sequences are (at least partially) curated based on
173 taxonomic and phylogenetic expertise (e.g., PR^2, Guillou et al. 2013; EukRef, http://eukref.org/).
174
175 **Descriptions, redescriptions, and nomenclature changes**
176 Taxonomic acts are regulated by the International Code of Zoological Nomenclature (ICZN
177 1999). For the description of new species and the redescription of insufficiently known species
178 of tintinnids, it is indispensable that the lorica information necessary for identification is
179 complemented by additional lorica and cytological features as detailed as possible (Box S1.2,
180 S1.3). Permanent material must be deposited in an acknowledged collection (ICZN 1999), and at
181 least SSU rDNA sequences should be submitted to public repositories (Lynn and Simpson 2009).
182 A combination of live observation and protargol staining reveals the majority of
183 species-specific morphological features in most ciliate groups and the latter provides permanent
184 slides for deposition (Foissner 2014). Low numbers and/or poorly stained cells often prevent

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185 accurate descriptions and redescriptions. It is difficult to predetermine a number of specimens to
186 study, yet morphometric data and statistical analyses should be grounded on enough specimens
187 to grant the best compromise between effort and accuracy. An adequate sample size avoids the
188 establishment of new species based on possibly atypical specimens and allows rough estimates
189 of the intraspecific variability; in this sense, it is also important to study several populations.
190 Species under scrutiny need careful comparison with congeners and other similar
191 species, including the discussion of resemblances and differences in morphological and
192 molecular characters. Currently, the scarce knowledge on intraspecific and interspecific
193 variability in cell morphology (only about 30 species have been studied cytologically, generally
194 based on up to 30 individuals from single populations; Agatha and Strüder-Kypke 2013) as well
195 as the lack of an absolute barcode gap in the commonly used molecular markers (Santoferrara et
196 al. 2015) makes difficult separating or lumping species unequivocally. Therefore, we encourage
197 comparing as many features as possible (e.g., multiple morphological and molecular parameters,
198 biogeography, ecophysiology), which also maximises the chance of identifying new diagnostic
199 characters. Synonymizations and other nomenclature changes should never be grounded on weak
200 evidence (e.g., gene phylogenies of species potentially misidentified by their lorica). Taxa
201 reclassifications should only be performed when the diagnosis can be improved by reliable
202 morphological and/or genetic synapomorphies from accurately identified specimens.
203
204
205 **CONCLUSIONS**
206 The 'taxonomic impediment', i.e., the decline in taxonomic knowledge together with the number
207 of classical taxonomists, limits the accuracy of species identification and the adequacy of species
208 descriptions and redescriptions (Wägele et al. 2011). At first glance, it seems desirable to
209 accelerate the pace of species investigations by adopting a "turbo-taxonomy" approach,
210 focussing on a few, easily accessible morphological features, which is particularly tempting
211 using the tintinnid lorica. However, in the long run, it might turn out that these rapidly produced
212 data (e.g., during species discovery or DNA barcoding) lack relevant information or cause errors,
213 thereby increasing confusion in taxonomic and ecological work.
214 Future directions in tintinnid studies should include the integral redescription of type
215 species, which are difficult to sample even in the type localities, but would enable taxonomic
216 rearrangements needed in several families and genera (Agatha and Strüder-Kypke 2014). Other
217 species of interest are the ones from comparatively less studied environments, such as the open
218 ocean, which are more challenging as their low abundances hamper the collection of sufficient
219 material and some of their features are more difficult to study during oceanographic expeditions.
220 But even the insufficiently known species that are abundant in coastal waters are worth
221 examination, because they might possess surprising morphological features and can provide
222 useful DNA barcodes. This information is crucial to build accurate, public databases of reference
223 sequences, especially in the context of current advancements that are allowing to tackle tintinnids
224 from an "-omics" perspective (Bachy et al. 2013; Keeling et al. 2014).
225 Complementary morphological, molecular, and ecological data are needed to provide
226 key insights, namely, a natural classification system, a reliable evolutionary model, and a deep
227 understating of biogeography and ecological roles (Fig. 1). To facilitate this aim, we provide
228 checklists and recommendations for data collection and evaluation (Box S1, S2, S3). The
229 collaboration of experts in different disciplines may be the most productive way to carry out such
230 integrated biodiversity studies.

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232
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238
239
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374 **FIGURE LEGEND**

375 **Figure 1.** Biodiversity studies in tintinnid ciliates. Species identification is still based almost
376 exclusively on lorica features such as the structure, shape, and size, especially of the oral
377 diameter (OD). Accurate determination is fundamental for subsequent steps such as (i)
378 estimation of species abundance and distribution for ecological studies, (ii) linkage of DNA
379 sequences to species for phylogenetic inference and the establishment of reference databases,
380 and (iii) the re-/description of species based not only on lorica characters, but also on cell
381 features recognizable in live and protargol stained material. Integration of morphological,
382 molecular, and ecological information (Supporting Information: Box S1, S2, S3) is needed to
383 provide a natural classification system, a reliable evolutionary model, and a deep understating of
384 biogeography and ecological roles. L, length.

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387 **SUPPORTING INFORMATION**

388 Box S1. Morphological data

389 Box S2. Molecular data

390 Box S3. Metadata and ecological data

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Journal of Eukaryotic Microbiology

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

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SUPPORTING INFORMATION

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

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Box S1. Morphological Data

1.1. Recommendations for species identification

1.1.1. Material: alive or preserved (Bouin's solution, Lugol's solution; Gifford and Caron 2000)
1.1.2. Investigation methods: light microscopy at least at 400 × magnification
1.1.3. Characters for identification: lorica features (see 1.2.6); when available, cell features (1.2.7)
1.1.4. Literature for identification: original description or authoritative redescription

1.2. Recommendations for descriptions and redescriptions

1.2.1. Material: alive and preserved with Bouin's solution (Foissner 2014)
1.2.2. Investigation methods: live observation and protargol impregnation yielding permanent slides (Foissner 2014)
1.2.3. Kind of specimens studied: morphostatic specimens (non-dividers); in dividers, only those characters that are not influenced by cell division
1.2.4. Number of randomly selected specimens studied for commonly variable characters: at least 10, in order to obtain a standard error of the arithmetic mean of not more than 25% and a confidence interval of at least 90%
1.2.5. Number of randomly selected specimens studied for highly variable characters: about 40
1.2.6. Lorica features (for further information, see Agatha et al. 2013)
 1.2.6.1. Shape. The details (e.g., teeth at the opening rim, windows, structures at the posterior process, and presence of a subterminal membrane) usually require the application of about 1000 × magnification and interference contrast optics; note that details are generally not visible in permanent slides
 1.2.6.2. Morphometrics: length (total and of particular portions); inner diameters of anterior and posterior openings and anterior constriction (the most reliable characters); number of spirals and rings; maximum width; width of posterior process; number and size of windows, ribs, fins, and teeth; size of pores and alveoli. Length and width can be measured at 400 × magnification, other details generally require 1000 × magnification Measurements are performed by means of a calibrated ocular or image analyser
 1.2.6.3. Structure (hyaline; agglutinated and hard; agglutinated and flexible; with hyaline collar and agglutinated bowl); it is easily visible in live and preserved material even at about 400× magnification
 1.2.6.4. Wall texture (e.g., solid, composed of alveoli or tubules, with spirals, rings, surface ridges); generally requires inspection at about 1000× magnification and with interference contrast optics; note that the texture might not be visible in permanent slides
 1.2.6.5. Ability to actively collapse the lorica opening; only visible in live material
 1.2.6.6. Lorica sac and closing apparatus, if present; most easily visible in live specimens
 1.2.6.7. Terminology: according to Agatha et al. (2013)
1.2.7. Cell features [IV – in vivo at 250–1000× magnifications, especially for cell features that shrink, swell, contract, or completely disappear with preservation; PI – protargol impregnation indispensable for revealing the ciliary pattern and nuclear apparatus; protargol can be produced in-house according to protocol of Pan et al. (2013); impregnation protocols according to Foissner (2014) and Song and Wilbert (2009)]. For species with agglomerated loricae, objections that attached particles might obscure the somatic ciliary pattern are unfounded provided that well-impregnated cells are available (e.g., Agatha and Riedel-Lorjé 2006; Foissner and Wilbert 1979).
 1.2.7.1. Size of cell proper and peduncle (IV and PI)
 1.2.7.2. Shape in extended and contracted state (IV), lateral projections that cause the lorica to collapse (IV)
 1.2.7.3. Macronucleus nodules: number, shape, and size (PI)
 1.2.7.4. Micronuclei: number, shape, and size (PI); occasionally not impregnated

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Box S1. Morphological Data – *Continued*

1.2.7.5. Structure of kinetids, especially in ventral, dorsal, posterior, second, and third kinety (PI); note that unciliated basal bodies might not be stained
1.2.7.6. Length of cilia, especially of elongated cilia in the dorsal and posterior kineties, in ventral organelles and tufts, and at the anterior kinety ends in the right and left ciliary fields (PI)
1.2.7.7. Right, left, and lateral ciliary fields: number and length (minimum and maximum) of the kineties (PI)
1.2.7.8. Posterior kinety: position and length; number of kinetids (PI)
1.2.7.9. Dorsal kineties: number, position, and length; number of kinetids (PI)
1.2.7.10. Ventral kinety: shape, position, and length; distance to the right ciliary field, number of kinetids (PI)
1.2.7.11. Second kinety: shape, position, and length; number of kinetids (PI)
1.2.7.12. Last kinety: shape and position (PI)
1.2.7.13. Kinety fragments (PI)
1.2.7.14. Ventral organelles: size, orientation, structure (PI)
1.2.7.15. Adoral zone of membranelles (PI): diameter, orientation in contracted specimens. Collar membranelles: total number, number of elongated membranelles extending into the buccal cavity. Buccal membranelles: number
1.2.7.16. Terminology: according to Agatha and Strüder-Kypke (2013)

1.2.8. Data to publish
1.2.8.1. For descriptions: species name (nov. spec.), diagnosis (features that distinguish the species from congeners), etymology
1.2.8.2. For redescriptions: synonyms, improved diagnosis
1.2.8.3. Detailed description including all observations (1.2.6 and 1.2.7)
1.2.8.4. Morphometric data (1.2.6.2 and 1.2.7) as a table
1.2.8.5. Molecular data (Box 2.1)
1.2.8.6. Metadata (Box 3.1)
1.2.8.7. For descriptions: comparison of new species with original descriptions or authoritative redescriptions of most similar species, including the particular illustrations used
1.2.8.8. For redescriptions: comparison with original description or previous redescriptions, including the particular illustrations used
1.2.8.9. Name of slide collection and accession number of slides

1.2.9. Illustrations
1.2.9.1. High-resolution micrographs at appropriate magnification to show the relevant details of cell and lorica. Several focal planes should be re-assembled by computer programs to display the ciliary pattern of one cell side
1.2.9.2. Drawings: (i) a live specimen based on free-hand sketches and mean measurements summarizes the available information, (ii) ventral and dorsal sides preferably of the same protargol-impregnated (type or neotype) specimen, using a drawing device such as a camera lucida; (iii) a two-dimensional scheme of the somatic ciliary pattern (kinetal map) based on the average morphometric data

1.2.10. Deposition of type and voucher material beyond Article 72.10. of the ICZN (1999): permanent slides containing specimens well-impregnated with protargol should be deposited in an acknowledged collection [e.g., Natural History Museum in London (Great Britain), Oberösterreichisches Landesmuseum in Linz (Austria), or National Museum of Natural History, Smithsonian Institution in Washington (USA)] and labelled following Foissner (2014)

1.2.11. Addition of nomenclatural acts to ZooBank in accordance with the criteria given in ICZN (2012)

1.3. Additional recommendations for descriptions and redescriptions

1.3.1. Material: clonal cultures may be established, although they are time-consuming and often not

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Box S1. Morphological Data – *Continued*

successful. Also, ciliate phenotype and genotype may change due to the accumulation of mutations under long exposure to adverse or uncommon culturing conditions (Day and Stacey 2007). Nevertheless, cultures are extremely useful in addressing issues such as intraspecific polymorphism within cell or life cycles and may thus help to avoid misidentifications and inaccurate species circumscriptions (Laval-Peuto 1981)

1.3.2. Investigation methods: beyond live observation and protargol impregnation, the use of scanning electron microscopy (SEM: Agatha and Tsai 2008; Foissner 2014) and transmission electron microscopy (TEM; Laval-Peuto 1975) is suggested
1.3.3. Data on ontogenesis, especially concerning the position of the oral primordium (PI)
1.3.4. Data on conjugation (PI)
1.3.5. Data on cyst morphology (IV, PI, SEM, TEM)
1.3.6. Lorica features: kinds and sizes of foreign particles (IV, SEM)
1.3.7. Cell features
    1.3.7.1. Length of cilia and collar membranelles (IV)
    1.3.7.2. Macronucleus nodules: position, size of nucleoli (PI)
    1.3.7.3. Somatic kineties: number of kinetids in each ciliary row, distances between each other and to the collar membranelles (PI)
    1.3.7.4. Oral ciliature: structure (SEM, TEM) and length (PI) of the bases (polykinetids) in the collar and buccal membranelles, including the elongated collar membranelles; shape and structure of the endoral membrane (PI, SEM, TEM); depth of the buccal cavity (IV, PI), number of collar membranelles in oral primordia of ventrally orientated middle or late dividers (PI)
    1.3.7.5. Tentaculoids, striae, accessory combs: shape, position, and size (IV, SEM)
    1.3.7.6. Capsules: arrangement (IV, SEM), ultrastructure (TEM)
    1.3.7.7. Fibres associated with the oral and somatic ciliature, including pharyngeal fibres (PI, TEM)
    1.3.7.8. Argyrophilic granules associated with the posterior and/or dorsal kinety or the collar membranelles (PI)
    1.3.7.9. Myonemes in the peduncle: shape, number, and size (PI, TEM)
    1.3.7.10. Cytopyge: position (IV)
    1.3.7.11. Contractile vacuole: shape, number, and position (IV, TEM)
    1.3.7.12. Cytoplasm: colour, special inclusions (IV)
1.3.8. Data to publish
    1.3.8.1. Morphometric data (IV, PI, SEM, TEM): analyses of more specimens; submission of raw data as excel file
    1.3.8.2. Comparison of insufficiently known species in redescriptions with other populations and similar species
    1.3.8.3. Illustrations: High-resolution videos (IV) and additional micrographs (IV, PI, SEM, TEM) as supplementary material
    1.3.8.4. List of synonyms: each synonym should be re-evaluated to reflect the authors' opinion
1.3.9. Deposition of voucher material for future morphological studies: Bouin-preserved material for protargol impregnations, further slides in a second repository

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Box S2. Molecular Data

2.1. Recommendations for DNA barcoding

2.1.1. Material: alive or preserved with Lugol's solution (non-acid formula; Auinger et al. 2008)
2.1.2. Investigation methods: Sanger sequencing from single cells or clonal cultures (Lynn and Pinheiro 2009)
2.1.3. Source of DNA for PCR amplification: extracted DNA (preferred over direct PCR on whole cells, which makes it impossible to sequence additional markers or to store genomic DNA)
2.1.4. Type of sequencing: direct or after cloning. Cloning is needed if the presence of other organisms (e.g., contamination, food items, parasites, symbionts) is known or suspected. Sequence enough clones to obtain the accurate tintinnid sequence
2.1.5. Markers: at least SSU rDNA. Complete or almost complete sequences should be obtained for descriptions, redescriptions, phylogenetics, and whenever is possible. For strict barcoding, the V4 region of SSU rDNA has been suggested by the CBOL Protist Working Group as a pre-barcode (Pawlowski et al. 2012)
2.1.6. Sequence quality: according to published standards (Hanner 2009)
 2.1.6.1. Sequence entire region in both forward and reverse directions
 2.1.6.2. Trim low quality regions (quality scores < 20 in chromatograms from Sanger sequencer) and primers
 2.1.6.3. Final sequence length should be close to the expected marker length
 2.1.6.4. Check very divergent sequences and discard contaminations or chimeras
 2.1.6.5. Manually inspect and exclude potential sequencing errors, which is particularly important for closely related species that usually differ in few nucleotides
2.1.7. Data to publish for each specimen whose DNA sequence is deposited in a publicly accessible repository (e.g., NCBI GenBank):
 2.1.7.1. Morphological data (Box 1.1)
 2.1.7.2. Morphometric data (Box 1.2.6.2) as a table
 2.1.7.3. Public repository accession number
 2.1.7.4. Metadata (Box 3.1)
 2.1.7.5. High-resolution micrographs of different focal planes or a high resolution video footage focussing through the loricae both at an appropriate magnification to show the taxonomically relevant details
 2.1.7.6. A comparison with the original description or authoritative redescription

2.2. Additional recommendations for molecular studies

2.2.1. Number of specimens sequenced: enough to reflect lorica plasticity of a hypothetical species
2.2.2. Cloning: to evaluate intra-individual variability (Gong et al. 2013). Sequence enough clones to support statistical analyses
2.2.3. Markers: LSU rDNA and/ or ITS1-5.8 rDNA-ITS2 are necessary for the discrimination of closely related species. The LSU rDNA is suggested as an alternative barcode for ciliates (Stoeck et al. 2014)
2.2.4. For species descriptions and redescriptions, inclusion of molecular apomorphies into taxon diagnosis
2.2.5. Storage of material for future molecular analyses: cells preserved in non-acid Lugol's solution (short storability), cells stored in absolute ethanol (moderate storability), DNA on Whatman FTA® collection cards (storability for many years), cryopreserved cells and long-term monoclonal cultures in culture collections, extracted genetic material in biobanks at ultra-low temperatures
2.2.6. Reference databases for molecular identification and phylogenetics: use continuously updated and curated sequence databases. Largely incomplete, low-quality, or potentially misidentified sequences should be excluded. For example, if very different sequences are attributed to the same species, the sequence linked to adequately published data should be given priority. Public reference databases should be carefully evaluated before use
2.2.7. See additional recommendations by Fontaneto et al. (2015)

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Box S3. Metadata and Ecological Data

3.1. Main data

3.1.1. Sampling: date, coordinates
3.1.2. Environmental conditions: water temperature, salinity
3.1.3. Preservation methods: fixative, final concentration
3.1.4. Cultures (if used): duration of cultivation prior to investigation, medium, culture conditions, food
3.1.5. Staining methods: preparation techniques
3.1.6. Molecular methods: DNA extraction and purification kits or protocols, primers used, PCR amplification and sequencing conditions, methods for alignments and phylogenetic inferences

3.2. Additional recommendations

3.2.1. Sampling: maps, pictures, and schemes (e.g., bathymetry chart) of sites as supplementary material
3.2.2. Site characteristics and additional environmental data: concentration of dissolved oxygen, pH, weather, wind; in freshwater species, size of pond or lake and mixing regime
3.2.3. Abundance: cell counts under inverted microscope (Utermöhl 1958). The Quantitative Protargol Stain (Montagnes and Lynn 1993) allows simultaneous quantitative and qualitative analyses of planktonic ciliates, but is time consuming
3.2.4. Biomass: estimations based on abundance (Box 3.2.3), lorica length and width (Box 1.2.6.2) or cell size (Box 1.2.7.1), and biovolume to carbon conversion factors [inferred from lorica size: Verity and Langdon (1984); inferred from size of preserved cell: Putt and Stoecker (1989)]
3.2.5. Other ecological information: associated species, content of food vacuoles, parasite infection, cyst formation, conjugation, etc.
3.2.6. Physiological data: growth rate, ingestion rate, food preferences, best culture conditions (e.g., optimal temperature)
3.2.7. Behaviour: swimming patterns, phototaxis, etc.
3.2.8. Geographic distribution and seasonality: critical revision of all previous reports to evaluate spatial and temporal patterns (e.g., Dolan et al. 2014; Saccà and Giuffrè 2013)

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