Morphological and Ribosomal DNA-based Characterization of Six Antarctic Ciliate Morphospecies from the Amundsen Sea with Phylogenetic Analyses

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18S; 28S; Antarctica; marine ciliates; planktonic ciliates; taxonomy; Tintinnida.

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ABSTRACT

We characterized six tintinnid ciliates from Antarctic waters using molecular markers and morphological traits: \textit{Amphorellopsis quinquealata}, \textit{Codonellopsis gaussi}, \textit{Cymatocylis convallaria}, \textit{Cy. calyciformis}, \textit{Cy. drygalskii}, and \textit{Laackmanniella prolongata}. The 100\% similarity in SSU-ITS1-5.8S rDNA-ITS2-partial LSU rDNA sequences among \textit{Cy. convallaria}, \textit{Cy. calyciformis}, and \textit{Cy. drygalskii} is supportive of synonymy. \textit{Codonellopsis gaussi} and \textit{L. prolongata} also showed high levels of similarity in SSU rDNA (99.83\%) and the D2 domain of LSU rDNA (95.77\%), suggesting that they are closely related. Phylogenetic analysis placed \textit{Cymatocylis} in the Rhabdonellidae, \textit{Amphorellopsis} in the Tintinnidae and \textit{L. prolongata}/\textit{Co. gaussi} within the Dictyocystidae.

THE Southern Ocean is known to harbor endemic tintinnid species (Petz et al. 2007; Pierce and Turner 1993). Among these, \textit{Codonellopsis gaussi}, \textit{Cymatocylis} spp., and \textit{Laackmanniella prolongata} have been found frequently in Antarctic and Subantarctic waters due to both significant biomass and distinctive lorica morphologies (e.g., Alder and Boltovskoy 1991a,b; Boltovskoy and Alder 1992; Boltovskoy et al. 1988; Dolan et al. 2012; Heinbokel and Coats 1984, 1986; Monti and Fonda Umani 1995; Petz et al. 1995; Thompson 2004; Thompson et al. 1999; Williams et al. 1994). Like other tintinnid ciliates, these Antarctic forms have been identified based on lorica morphology. The possibility of synonymy among Antarctic species due to plasticity in lorica morphology has been repeatedly raised and discussed extensively (Alder and Boltovskoy 1991b; Boltovskoy and Alder 1992; Boltovskoy et al. 1990; Petz et al. 1995; Wasik and Mikolajczyk 1992). Although many studies have investigated Southern Ocean tintinnids, lack of accuracy and confidence in the species identities has complicated ecological and biogeographical studies of Antarctic tintinnid ciliates (Alder and Boltovskoy 1991b; Boltovskoy et al. 1990; Petz et al. 1995; Wasik and Mikolajczyk 1992). Since 2002 (Snoeyenbos-West et al. 2002), tintinnid ciliates have been investigated using molecular approaches based on small subunit ribosomal DNA (SSU rDNA) (Agatha and Struder-Kypke 2007, 2012; Bachy et al. 2012, 2013; Gao et al. 2009; Kim et al. 2010; Li et al. 2013; Santoferrara et al. 2012, 2013; Struder-Kypke and Lynn 2003, 2008; Xu et al. 2012). Consequently, tintinnids are now well-represented among the choreotrich ciliates in phylogenetic trees. However, because SSU rDNA is highly conserved, even hypervariable regions do not contain species-specific markers (Santoferrara et al. 2013). Recently, the internal transcribed spacer (ITS) regions of the rDNA, which are less
conserved than the SSU rDNA, have been used to study tintinnid phylogeny (Bachy et al. 2012, 2013; Snoeyenbos-West et al. 2002). The ITS region has also been used to study gene flow and distinguish cryptic species of oligotrichs (Katz et al. 2005; McManus et al. 2010). In a recent study, the phylogeny of tintinnids based on ITS and 5.8S rDNA regions corresponded well with SSU rDNA-based phylogeny, except for the placement of the genus Tintinnidium (Bachy et al. 2012). In studies of other groups of ciliates, large subunit ribosomal DNA (LSU rDNA) is commonly used for phylogenetic analysis as well as SSU rDNA (Gong et al. 2007; Hewitt et al. 2003; Nanney et al. 1998). Nanney et al. (1998) suggested that the divergent 2 (D2) domain of LSU rDNA can be used to identify cryptic species in Tetrahymena, Paramecium, and Colpoda. More recently, Santoferrara et al. (2013) supported the use of LSU rDNA as a genetic maker for tintinnid ciliates.

In this study, we employed a single-cell PCR method and obtained SSU, 5.8S, partial LSU including the D2 domain and the ITS region of ribosomal DNA sequences from six Antarctic forms described previously as individual species. Protargol staining and measurement of lorica shapes were also conducted for morphological characterization. The synonymy and phylogeny of these species are discussed based on both the morphological and molecular data.

MATERIALS AND METHODS

Sample collection

Samples were collected using a 20-μm plankton net from the Amundsen Sea (65°68′S, 111°27′W) on board the RV Aron in December 2010. Sea water temperature and salinity ranged from −1.68 °C to 1.09 °C (average −0.81 ± 0.85 °C) and 33.13–34.20 psu (average 33.80 ± 0.30 psu), respectively. On board, part of the sample was fixed in 80% ethanol at −80 °C until further processing for molecular analysis. The remainder of the sample was fixed in 6% Bouin’s solution for subsequent morphological studies.

Cell isolation, PCR amplification, and sequencing

Preserved material in ethanol was transferred to autoclaved distilled water (DW). Individual cells were then selected under a dissecting microscope and deposited onto a slide using a Pasteur pipette. Each cell was isolated with a new pipette to prevent contamination. Each cell was rinsed at least five times with autoclaved DW to remove other organisms. Images of isolated cells were obtained using a microscope equipped with a digital camera to record lorica morphology. After imaging, cells were transferred to individual PCR tubes containing 20 μl of DW. Without use of a DNA extraction step, the PCR mixture was transferred to the PCR tube with the isolated cells, resulting in a 50-μl total volume. The TaKaRa LA Taq polymerase was used according to the manufacturer’s instructions with EuKA and Rev2 primers (Table S1). PCR amplifications for the SSU-partial LSU rDNA gene were modified from Jung et al. (2011) with the following conditions: denaturation for 2 min at 94 °C, followed by 37 cycles of denaturation for 30 s at 95 °C, annealing for 40 s at 50 °C, extension for 4 min at 72 °C, and a final extension at 72 °C for 10 min. The PCR product was purified using the Gel Extraction Kit (QIAGene, Chatsworth, CA) and sequenced on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA). Four or five additional inter-nal primers were used to assist in sequencing the SSU-partial LSU rDNA sequences (Table S1).

DNA sequence comparisons

Sequence similarities were determined by comparison with DNA sequences of Antarctic species from this study. Sequences were aligned using CLUSTAL X v. 1.81 (Jeanmougin et al. 1998). Intra- and inter-specific similarities were investigated by comparing the DNA similarities of SSU rDNA, partial LSU rDNA, the D2 domain of LSU rDNA and ITS 1 using the Phydit program v. 3.1 (Chun 2001). The D2 domain of LSU rDNA was identified following the guidelines of Engberg et al. (1990).

Phylogenetic analysis

Sequences of SSU and LSU rRNA were used for phylogenetic tree construction. To represent the phylogeny of six Antarctic species, 81 SSU rRNA gene sequences of tintinnid ciliates were retrieved from the NCBI database (Fig. 40). Most tintinnid sequences were included, but partial sequences shorter than 1,429 bp were excluded. To compare the phylogenetic trees based on SSU rDNA and partial LSU rDNA, 29 LSU rDNA gene sequences of tintinnid ciliates were retrieved from the NCBI database following the method of Santoferrara et al. (2012). Each data set was aligned using SILVA Incremental Aligner (SINA) v. 1.2.11 (Pruesse et al. 2012) and refined manually using the Bioedit program v. 7.1 (Hall 1999). Hypervariable regions that could not be aligned unambiguously were removed. After the ends of the alignments were trimmed, separate phylogenetic analyses were performed for SSU rRNA (1,359 bp) and LSU rRNA (506 bp). Six species from the subclasses Choreotrichia (order Choreotrichida), Oligotrichia, and Stichotrichia were used as outgroups. The program MrModeltest v. 2 (Nylander 2004) selected the GTR + I + G as the best model using Akaike information criterion, which was used for both Bayesian and maximum-likelihood (ML) inference. The Bayesian tree was constructed from an output of 6,000 trees generated by MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) with 6,000,000 cycles for the Markov Chain Monte Carlo algorithm and sampling every 1,000th generation. Stationary likelihood scores were determined by plotting the lnL against the generation. The first 1,500 trees below the observed stationary level were discarded as burn-in. A ML tree was constructed with the PhyML v. 2.4.4 program (Guindon and Gascuel 2003). Reliability of internal branches was assessed using the non-parametric
bootstrap method with 1,000 pseudoreplicates. TreeView v. 1.6.6 (Page 1996) and MEGA v. 4.0 (Tamura et al. 2007) were used to visualize tree topology.

**Morphological observation**

Lorica morphology was examined using light microscopy. Cells were picked randomly from fixed samples for morphological examination of their lorica using a Sedgwick–Rafter chamber. Observations and drawings of stained specimens were performed at 640X and 1,600X with a camera lucida. We followed the terminology proposed by Agatha and Riedel-Lorjé (2006). Protargol staining was conducted following the quantitative protargol staining method of Montagnes and Lynn (1987). Unfortunately, in our protargol preparations, it was not possible to clearly determine the somatic ciliary patterns in Cymatocylis spp. and Amphorellopsis quinquealata. For species identification, the original descriptions of Laackmann (1907, 1910), and the commonly employed monographs of Kofoid and Campbell (1929, 1939) and Petz et al. (1995), were used.

**RESULTS**

**Description of six Antarctic species**

Morphometric analyses of the loricae for the six taxa are presented in Table 1. We were only partially successful in

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Measurements in μm. 
M = median; Max = maximum; Min = minimum; n = number of individuals investigated; CV = coefficient of variation; SD = standard deviation; x = arithmetic mean; SE = standard error of arithmetic mean.

Table 1. Morphometric data of Cymatocylis convallaria (1st line), Cy. calyciformis (2nd line), Cy. drygalskii (3rd line), Codonellopsis gaussi (4th line), Laackmanniella prolongata (5th line), and Amphorellopsis quinquealata (6th line)
using protargol staining to characterize the infraciliature; complete mapping of the ciliature was not possible with our preparations. We were able to enumerate oral membranelles and somatic ciliature for *Laackmanniella prolongata* and *Codonellopsis gaussi*.

**Cymatocylis convallaria Laackmann, 1910 (Table 1 and Fig. 1, 12, 13)**

*Cymatocylis convallaria* Laackmann, 1910; Deutsch. Südpolar-Exp., 11:383, pl. 33, fig. 5, pl. 43, fig. 1–4.

*Cymatocylis affinis* Laackmann, 1910; Deutsch. Südpolar-Exp., 11:384, pl. 43, fig. 5–15.


The infraciliature structure of this species was revealed using protargol impregnation by Wasik and Mikołajczyk (1994) and Petz et al. (1995). *Cymatocylis affinis* and *Cy. convallaria* are now known to have identical patterns of infraciliature (Petz et al. 1995; Wasik and Mikołajczyk 1992), and were consequently termed *Cy. affinis/convallaria* and is now called *Cy. convallaria* by

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**Figure 1–7** Lorica morphology of Antarctic species. 1. *Cymatocylis convallaria*. 2. *Cymatocylis calyciformis*. 3. *Cymatocylis drygalskii*. 4. *Laackmanniella prolongata*. 5, 6. *Codonellopsis gaussi*. 7. *Amphorellopsis quinquealata*. Scale bars: 100 μm (Fig. 1–3) and 50 μm (Fig. 4–7).
Petz et al. (1995). Additional synonyms have been introduced in Petz et al. (1995), and Petz (2005), but omitted in the present article because the synonyms are based only on lorica morphology or the explanation of synonymizations are absent.

**Description of the Amundsen population**

The lorica is hyaline and bell-shaped. The posterior end is pointed (Fig. 1, 12) or having a short process 10–20 \( \mu \)m in length (Table 1). The anterior end of the lorica possesses an inner collar with fine teeth (4–5 \( \mu \)m in length) and the outer collar is bent outward (Fig. 1, 13). The lorica wall has a polygonal structure with \( \sim \)4–5 \( \mu \)m diagonals, which becomes finer (the diagonals become smaller in size) toward the posterior portion of the lorica (Fig. 13). The overall lorica size is 140–150 \( \mu \)m in length with a width of 85–110 \( \mu \)m (Table 1). The lorica opening diameter ranges from 90 to 95 \( \mu \)m (Table 1). Two globular macronuclei have been observed.

**Comparison with original description**

Laackmann (1910) described the lorica size of *Cy. convallaria* as 110–140 \( \mu \)m in length and 95–120 \( \mu \)m in width. The lorica opening diameter of *Cy. convallaria* was not provided in Laackmann (1910), but is estimated as \( \sim \)85–100 \( \mu \)m based on illustrations. Laackmann (1910) reported the lorica size of *Cy. affinis* as 120–170 \( \mu \)m in length and 90–110 \( \mu \)m in width, which has been regarded as *Cy. convallaria* by Petz et al. (1995), and Wasik and Mikolajczyk (1994), by comparing the infraciliature structure. The lorica opening diameter of *Cy. affinis* is estimated to be 90–110 \( \mu \)m based on the illustrations of Laackmann (1910). Our specimens correspond well with the original descriptions of *Cy. affinis* and *Cy. convallaria*.

**Comparison with similar species**

Petz (2005) considered *Cymatocylis parva* as a synonym of *Cy. convallaria*. Laackmann (1907) described *Cy. parva*...
as having a lorica opening diameter of 47–67 μm, distinctly different from the original Cy. convallaria description and our population.

**Cymatocylis calyciformis** (Laackmann 1907)

Laackmann, 1910 (Table 1 and Fig. 2, 10, 11)

Cytarocolis calyciformis Laackmann, 1907, Zool. Anz., 31:236, fig. 3.

Cymatocylis calyciformis Laackmann, 1910, Deutsch. Südpolar-Exp., 11:391, pl. 36, fig. 4; pl. 42, fig. 12.


The infraciliature structure of this species was reported using protargol impregnation (Petz et al. 1995). Additional synonyms have been introduced in Petz et al. (1995) and Petz (2005), but omitted in the present article because the synonymizations were not justified.

**Description of the Amundsen population**

The lorica is hyaline and cup-shaped with a 55–145 μm long posterior process (Table 1 and Fig. 2, 10). The anterior end of the lorica is formed of an inner collar with fine teeth 2–5 μm in length and an outer collar bent outward (Table 1 and Fig. 2, 11). The lorica has a polygonal structure with ~4.5 μm diagonals, which is finer toward the posterior portion of the lorica (Fig. 11). The overall lorica dimensions are 215–310 μm in length and 90–110 μm in width (Table 1). The lorica opening diameter ranges from 80 to 100 μm (average 95 μm, Table 1). Two globular macronuclei are also observed.

**Comparison with the original description**

The lorica of Cy. calyciformis was originally described as 440 μm in length and 106 μm in width, with a 133-μm lorica opening diameter and a bowl length of 266 μm (Laackmann 1907). Although the lorica shapes of our specimens and that described originally for Cy. calyciformis fit perfectly, the lorica opening diameter, generally considered a preserved character, does differ compared to that given by Laackmann, respectively, 133 μm and 95 μm. However, if the lorica width given by Laackmann (1910) was measured at the anterior lorica end, it possibly includes the outer collar of ~10 μm width, which is not considered in our measurements. Furthermore, there is considerable inconsistency among Laackmann’s descriptions. Laackmann in 1910 reported the lorica size of Cy. calyciformis as 400–520 μm in length and 150 μm in width. He supplied two illustrations of Cy. calyciformis; the illustration of fig. 12 in plate 42 of Laackmann (1910) is ~120 μm, based on the magnification scale given, and the another one, fig. 5 in plate 36 is ~125 μm. Notably, other workers have also found specimens conforming to the general morphology of Cy. calyciformis to have an oral diameter of 95–102 μm (e.g. Fernandes 1999; Sassi and Melo 1993). Therefore, we identified our specimens as Cy. calyciformis.
buccal membranelle (Fig. 22, 23, 27). Complex ciliary patterns (Agatha and Strüder-Kypke 2012) are observed, which have one monokinetidal ventral, one dikinetidal dorsal, and one dikinetidal posterior kinety, as well as a right, left, and lateral ciliary field (Fig. 22, 23, 29, 30, 32, 33). One dorsal kinety originates near a first kinety of the right ciliary field, curves toward the left ciliary field and extends the posterior part. The posterior kinety was observed below the left ciliary field. We observed ~21–26 somatic kineties; ~9–11 in the lateral ciliary field, ~5–8 in the left ciliary field, and ~5–9 in the right ciliary field (Fig. 28, 29, 31). The lateral ciliary field is composed of densely spaced monokinetids. The kineties of right and left fields are composed of monokinetids and one anterior dikinetid.

Comparison with the original description

Laackmann (1907) described the lorica of *Codonellopsis gaussi* as 155 μm in length, 54 μm in maximum width, with a 40-μm loric opening diameter. Later Laackmann (1910) gave the dimensions as 140–180 μm in length, 40–60 μm in width, with a 30–40 μm opening diameter. Our specimens corresponded with the overall ranges reported by Laackmann (1907, 1910).

Comparison with similar species

*Codonellopsis glacialis* has been described as likely conspecific with *Co. gaussi* (Balech 1958, 1973). Petz et al. (1995) reported the infraciliature of a Weddell Sea population of *Co. glacialis* to consist of 18–19 collar membranelles and 25–29 somatic kineties. The dorsal and posterior kineties of *Co. glacialis* were incorrectly named as a ventral and dorsal kinety, respectively, in Petz et al. (1995), but these kineties were clearly shown in the illustration. The position of the dorsal and posterior kineties of *Co. glacialis* fit well with our *Co. gaussi*. Our Amundsen Sea population of *Co. gaussi* overlapped with the Weddell Sea population of *Co. glacialis* in infraciliature characteristics (~17 collar membranelles, ~21–26 somatic kineties and the ciliary pattern), supporting the synonymy of *Co. gaussi* and *Co. glacialis*. However, more information, especially molecular data for *Co. glacialis*, is required.

*Laackmanniella prolongata* (Laackmann, 1907) Kofoid & Campbell, 1929 (Table 1 and Fig. 4, 15, 16, 25, 26, 34–39)

*Codonella prolongata* Laackmann, 1907, Zool. Anz., 31:239, fig. 11.
Figure 27–33 Micrographs of *Codonellopsis gaussi* after protargol impregnation. 27. Ventral view showing buccal cavity. 28. Lateral view showing lateral and left ciliary field. 29. Dorsal view showing dorsal kinety, left and right ciliary field. 30. Right ciliary field. 31. Dorsal view showing dorsal kinety. 32. Macronuclei. Ventral view. Arrow head marks the micronuclei. 33. Dorsal view showing dorsal and posterior kinety. BM = buccal membranelles; DK = dorsal kinety; F, fibers; LA = lateral ciliary field; LF = left ciliary field; Ma = macronuclei; PK = posterior kinety; RF = right ciliary field. The micrographs have been modified manually using Adobe Photoshop program with burning and sharpness tool. The original images are included in Fig. S12–S18.
Figure 34–39. Micrographs of *Laackmanniella prolongata* after protargol impregnation. **34.** Dorsal view showing dorsal kinety. **35.** Ventral view showing ventral kinety, lateral, and left ciliary field. **36.** Macronuclei and micronuclei. **37.** Ventral view of oral primordium. **38.** Dorsal view of dorsal kinety and right ciliary field. **39.** Ventral view showing posterior kinety. DK = dorsal kinety; F = fibers; LA = lateral ciliary field; LF = left ciliary field; Ma = macronuclei; Mi = micronuclei; PK = posterior kinety; RF = right ciliary field; VK = ventral kinety. The micrographs have been modified manually using Adobe Photoshop program with burning and sharpness tool. The original images are included in Fig. S19–S24.
Leprotintinnus prolongatus Laackmann, 1910, Deutch. Süd-polar-Exp., 11:403, pl. 46, fig. 10–12, pl. 48, fig. 5–7.


This species has previously been placed in diverse genera (Kofoid and Campbell 1929; Laackmann 1907, 1910). Additional synonyms have been introduced in Petz (2005), but omitted in the present article because information on synonymization is lacking.

Description of the Amundsen population

The lorica is bipartite and the anterior collar portion is tubular and hyaline with several spiral turns (Fig. 4, 15, 16). A posterior portion, also cylindrical, is agglutinated with mineral particles or many diatoms and tapers to an open posterior end (Fig. 4, 15, 16). The lorica is 145–214 μm in length and 30–40 μm in width (Table 1). The collar length of the lorica is 50–120 μm (Table 1). The opening diameter of the lorica is 32–40 μm (Table 1). The bowl length of the lorica ranges from 65 to 127 μm (Table 1). The posterior opening diameter of the lorica is 12–30 μm (Table 1). Four macronuclei and two micronuclei are observed (Fig. 25, 26). Most complex ciliary pattern (Agatha and Strüder-Kypke 2012) are observed, which have one monokinetid ventral, one dikinetid dorsal, and one dikinetid posterior kinety, as well as a right, left, and lateral ciliary field (Fig. 25, 26, 34, 35, 38). The posterior kinety was observed below the right ciliary field near the ventral kinety and extends straight toward the posterior section (Fig. 39). In a dividing cell, we noted a ventral kinety which curves along the margin of the oral primordium (Fig. 37). There are ~27 somatic kineties; ~10 in the lateral ciliary field, ~11 in the left ciliary field and circa six in the right ciliary field. The lateral ciliary field is composed of densely spaced monokinetids. The kineties of right and left fields are composed of monokinetids and one anterior dikinetid.

Comparison with the original description

Laackmann (1907) described Laackmanniella prolongata as having a lorica 308 μm in length and with a 40-μm opening diameter. Later, Laackmann (1910) reported the lorica size to range from 175 to 310 μm in length and 30–50 μm in width. The length of our specimens is smaller than that mentioned by Laackmann (1907), but overlaps with the range given by Laackmann (1910; 145–214 μm vs. 175–310 μm). The bowl length of our specimen is also smaller than the original description, which is estimated from the illustration (65–127 μm vs. ca. 140 μm); however, the lorica opening diameter corresponds with the records of Laackmann (1907, 1910). Also, Laackmann (1910) reported a short lorica in Leprotintinnus prolongata forma ventricosa of 140–250 μm in length and 50–55 μm in width, which was collected between February and April. The specimen of this population is similar in length to those of our population, but had a wider opening diameter. The differences in the lorica length might represent different developmental stages, variable collar lengths, or (such as Cymatoclysis) seasonal differences.

Comparison with similar species

There are only two species in Laackmanniella: L. naviculaefera, and L. prolongata. L. naviculaefera differs from L. prolongata in having a lorica shorter in overall length and showing a distinctive bulged bowl (Laackmann 1907). Although, L. prolongata is considered a synonym of L. naviculaefera by most workers (Alder 1999; Balech 1947, 1957; Hada 1970; Petz 2005), there is no infraciliature structure or DNA sequence of L. naviculaefera to compare with L. prolongata. In this article, our specimen is identified as L. prolongata based on the original description by Kofoid and Campbell (1929).

Amphorellopsis quinquealata (Laackmann, 1907)

Balech, 1971 (Table 1 and Fig. 7, 14)


Tintinnus quinquealatus Laackmann, 1910, Deutsch. Süd-polar-Exp., 11:412, pl. 47, fig. 13, 14; pl. 48, fig. 8.


This species has been previously placed in diverse genera (Balech 1971; Kofoid and Campbell 1929, 1939; Laackmann 1907), and is identified by its current name, A. quinquealata, according to Petz (2005).

Description of the Amundsen population

The lorica is hyaline without any agglutinated particles (Fig. 7, 14). The overall form is an elongate chalice-shape with five well-developed wings, which spiral down from the anterior marginal edge to the posterior end. The bowl (excluding wings) is a conical. The lorica is 140–210 μm in length and the opening diameter is 40–48 μm (Table 1). In the oral ciliature, we observed ~19–21 collar membranelles. Two globular macronuclei are apparent (Table 1). Laackmann (1910) recorded two micronuclei in this species, but the micronuclei were not visible in our population.

Comparison with the original description

Laackmann (1907) described the lorica of A. quinquealata as 190–206 μm in length and 46–57 μm in width. Later, Laackmann (1910) reported the lorica of this species to range between 180 and 215 μm in length with a lorica opening diameter of 40–45 μm. Our specimens are slightly smaller and more variable in lorica length, but have a similar lorica opening diameter (40–48 vs. 46–57 μm or 40–45 μm; Laackmann 1907, 1910).
Table 2. Fourteen tintinnid individual cells sequenced: lengths of SSU, ITS1, 5.8S, ITS2, and partial LSU rDNA sequences

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<th>Species (isolate number)</th>
<th>Total length (bp)</th>
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<th>ITS1-5.8S-ITS2</th>
<th>Partial LSU (D2 domain)</th>
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Similarities of rDNA sequences from six Antarctic species

Table 2 summarizes the sequence data obtained for the 14 individual cells (Fig. S1–S11) deposited in GenBank under accession numbers JQ924046–JQ924059. Excluding Cy. drygalskii, two to four cells of each species were isolated and sequenced (Table 2). Table 3 shows the similarities of the SSU and partial LSU rDNA sequences among the cells. The total length of these sequences was ~3,000 bp, including the D2 domain, which is known to be a variable region of LSU rDNA. For a morphospecies, we found 100% similarity in both SSU and LSU rDNA sequences (Table 3). We also found no variation among the three Cymatocylis forms (Cy. convallaria, Cy. calyciformis and Cy. drygalskii) in SSU, ITS1, 5.8S, ITS2, and partial LSU rDNA sequences (Tables 3, 4; ITS2 data not shown). Except for the 100% similarity within the three Cymatocylis species, the minimum interspecific deviations in the SSU rDNA sequences were 0.17% for Codonellopsis gaussi and Laackmanniella prolongata (Table 3, 5) and 1.13% in the partial LSU rDNA sequences. Despite the distinct lorica morphologies, Co. gaussi and L. prolongata were very closely related in terms of the similarity of their SSU rDNA sequences. Notably, the dissimilarities of the ITS 1 sequences and the D2 domains of the LSU rDNA were 4.81% and 4.23% between L. prolongata and Co. gaussi, respectively (Table 5).

Phylogenetic analyses

The Bayesian and ML trees based on SSU and partial LSU rDNA sequences showed that the six new sequences of Cymatocylis, Laackmanniella, Codonellopsis, and Amphorellopsis are well placed in the Tintinnida (Fig. 40). Three Cymatocylis species were clustered together within the Rhabdonellidae in all phylogenetic analyses (Fig. 40). The placement of Cymatocylis within the Rhabdonellidae had high supporting values (95% and 89% in BI trees based on SSU and partial LSU rDNA sequences). Laackmanniella prolongata and Codonellopsis gaussi were clustered together in the phylogenetic tree within the cluster of the Dictyocystidae, Stenosemella nivalis, and S. pacifica. The placement of L. prolongata and Co. gaussi in this cluster had only 59% and 58% supporting values in BI and ML trees based on SSU rDNA, respectively. In the BI and ML trees based on the LSU rDNA, L. prolongata and Co. gaussi clustered with Tintinnopsis parvula, which might be due to the lack of LSU rDNA sequences available for Dictyocystidae. The sequence of Amphorellopsis quinquealata was placed in the Tintinnidae, but the sequences clustered with Salpingella, not with Amphorellopsis acuta in the phylogenetic tree (Fig. 40). To classify these groups, sequences of more species, as well as morphological studies of the loria and somatic ciliary pattern, are required.

The trees based on partial LSU rDNA sequences were mostly in agreement with those based on SSU rDNA sequences, excluding the placement of Tintinnopsis parvula (Fig. 40). This is noteworthy because according to Sartoferara et al. (2012) a single T. parvula was the source of both the SSU and LSU rDNA sequences.

DISCUSSION

Morphological comparison of the Codonellopsis gaussi and Laackmanniella prolongata

Despite their distinct loria morphologies, Codonellopsis gaussi and Laackmanniella prolongata are closely related to the terms of genetic similarity. Also, they share the most complex ciliary pattern (Agatha and Strüder-Kypke 2007). However, the location of the posterior kinetics differs between the two species (below the left ciliary field vs. right ciliary field for Co. gaussi and L. prolongata, respectively). The taxonomic significance of differences in the position of the posterior kinety has not yet been considered (i.e., Agatha and Strüder-Kypke 2013). However, the infraciliature structure overall is well known to be a conserved character and the different positions of the
Table 3. Similarities of SSU and partial LSU rDNA sequences from six Antarctic species (%)

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Partial LSU rDNA (804 bp)

Shading is used to allow comparisons between individuals of the same species. Bold is used to highlight the similarity within morphospecies.
posterior kinety are considerable. Therefore, while these two species are closely related, genetically, they may be considered as separate species.

Phylogenetic analyses of tintinnid species

Our sequences were placed at positions that differed from the traditional, mostly lorica morphology-based, classifications (Fig. 40), which is similar to many recent studies (e.g., Bachy et al. 2012, 2013; Santoferrara et al. 2012, 2013). One of the disagreements between morphology and molecular phylogeny was resolved using the detailed morphological observation of Codonella, Codonaria, and Dictyocysta by Agatha (2010). The presence of a lorica sac in these three genera is suggestive of a close relationship, which was later supported by SSU rRNA phylogenies. Agatha and Strüder-Kypke (2012) united Codonaria, Codonella, and Codonellopsis in the family Dictyocystidae based on their morphological characteristics and molecular phylogeny.

In the phylogenetic trees based on SSU rDNA, Codonellopsis gaussi clustered with Laackmanniella prolongata within Dictyocystidae (Fig. 40). Notably, we found no evidence of a lorica sac in either form. Because of the genetic similarities, we suggest that Co. gaussi and L. prolongata should be united in one genus. However, further studies are required using live observation or scanning electron microscopy, including a detailed lorica structure comparison of these two species with other Codonellopsis species to place Co. gaussi and L. prolongata appropriately.

The placement of Cymatocylis species in the phylogenetic tree suggests that the familial affiliation of this species is not correct (Fig. 40). Based on the phylogenetic analysis, Cymatocylis does not group with Favella of Ptychocyclidae, but instead with Schmidingerella of Rhabdonellidae. Schmidingerella was recently established as a second cluster of Favella in the gene tree by Agatha and Strüder-Kypke (2012) based on morphological observations of the lorica ultrastructure and somatic ciliary pattern. Cymatocylis species are grouped with Schmidingerella and Metacylis; however, this is supported only by the BI tree based on SSU rDNA (Fig. 40). Laval-Peuto and Brownlee long ago argued that to correctly classify tintinnid species various approaches should be employed such as observation of cytology, and even non-morphological characteristics such as ecological and behavioral data (Laval-Peuto and Brownlee 1986). Recently, several studies have discussed the usefulness of combined molecular

Table 4. Similarities of ITS1 and D2 domain of LSU rDNA sequences from six Antarctic species (%)

<table>
<thead>
<tr>
<th>Species</th>
<th>T99</th>
<th>T100</th>
<th>T101</th>
<th>T102</th>
<th>T103</th>
<th>T104</th>
<th>T105</th>
<th>T106</th>
<th>T107</th>
<th>T108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cymatocylis calyciformis</td>
<td>–</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>89.11</td>
<td>91.09</td>
<td>90.74</td>
<td>76.24</td>
<td>76.24</td>
</tr>
<tr>
<td>Codonellopsis gaussi</td>
<td>100</td>
<td>–</td>
<td>100</td>
<td>100</td>
<td>91.09</td>
<td>91.09</td>
<td>91.09</td>
<td>91.09</td>
<td>76.24</td>
<td>76.24</td>
</tr>
<tr>
<td>Laackmanniella prolongata</td>
<td>95.19</td>
<td>95.19</td>
<td>76.24</td>
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</table>

D2 domain (193 bp)

Shading is used to allow comparisons between individuals of the same species. Bold is used to highlight the similarity within morphospecies.
Figure 40 Bayesian trees based on small subunit rRNA gene (left) and large subunit rRNA gene (right) sequences showing the relationships between six Antarctic tintinnid species (bold) and other tintinnid ciliates. Numbers at the nodes represent support values in the following order: Bayesian posterior probabilities using the MrBayes algorithm (BI) and bootstrap values from maximum likelihood (ML) analyses as % of 1,000 replicates. Asterisk (*) denotes nodes with full bootstrap support in all algorithms. A hyphen (-) represents support values < 50% and disagreement between BI and ML at a given node.

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analysis, cytological studies, and observation of lorica ultrastructure (Agatha 2010; Agatha and Strüder-Kypke 2012). All these lines of evidence will likely be needed to unambiguously place Cymatoclysis among tintinnids.

In conclusion, molecular markers provide vital support in identifying tintinnids, but should continue to be combined with morphological observations. Disagreements between molecular and morphological phylogenies suggest a need to employ a variety of genetic markers (including perhaps ITS2 secondary structure) and morphological features beyond those of the loricae traditionally used for identification and classification. Even the biometric approaches for objective classification of lorica types (e.g. Williams et al. 1994) have proven inadequate indicating a need for the identification and use of additional morphological characteristics, those neglected in traditional classifications. Studies of morphology and ecology coupled with molecular establish more reliable hypothesis about tintinnid evolution and to circumscribe tintinnid species properly.

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**SUPPORTING INFORMATION**

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


**Fig. S12–S18.** Original image of Fig. 27–33. Micrographs of *Codonellopsis gaussi* after protargol impregnation. S12. Ventral view showing buccal cavity. S13. Lateral view showing lateral and left ciliary field. S14. Dorsal view showing dorsal kinety, left and right ciliary field. S15. Right ciliary field. S16. Dorsal view showing dorsal kinety. S17. Macronuclei. Ventral view. Arrow head marks the micronuclei. S18. Dorsal view showing dorsal and posterior kinety. BM = buccal membranelles; DK = dorsal kinety; F = fibers; LA = lateral ciliary field; LF = left ciliary field; Ma = macronuclei; PK = posterior kinety; RF = right ciliary field.

**Fig. S19–S24.** Original image of Fig. 34–39. Micrographs of *Laackmanniella prolongata* after protargol impregnation. S19. Dorsal view showing dorsal kinety. S20. Ventral view showing ventral kinety, lateral and left ciliary field. S21. Macronuclei and micronuclei. S22. Ventral view of oral primordium. S23. Dorsal view of dorsal kinety and right ciliary filed. S24. Ventral view showing posterior kinety. DK = dorsal kinety; F = fibers; LA = lateral ciliary field; LF = left ciliary field; Ma = macronuclei; Mi = micronuclei; PK = posterior kinety; RF = right ciliary field; VK = ventral kinety.

**Table S1.** Primers used for DNA sequencing.