Phylogeny of two poorly known ciliate genera (Ciliophora, Heterotrichea), with notes on the redefinition of Gruberia uninucleata Kahl, 1932 and Linostomella vorticella (Ehrenberg, 1833) based on populations found in China

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Abstract

Background

Heterotrichous ciliates are common members of microeukaryote communities which play important roles in the transfer of material and energy flow in aquatic food webs. This group has been known over two centuries due to their large body size and cosmopolitan distribution. Nevertheless, species identification and phylogenetic relationships of heterotrichs remain challenging due to the lack of accurate morphological information and insufficient molecular data.

Results

The morphology and phylogeny of two poorly known heterotrichous ciliates, Gruberia uninucleata Kahl, 1932 and Linostomella vorticella (Ehrenberg, 1833) Aescht in Foissner et al., 1999, were investigated based on their living morphology, infraciliature, and small subunit (SSU) rDNA sequence data. Based on a combination of previous and present studies, detailed morphometric data and the improved diagnoses of both species are supplied here. In addition, molecular data of the two species are reported for the first time. Phylogenetic analyses based on SSU rDNA sequence data support the generic assignment of these two species.

Conclusions

Two insufficiently studied species, G. uninucleata and L. vorticella, are redefined using state-of-the-art methods. Previous reports on these two species are re-evaluated in the light of present findings.

Background

Members of the ciliate class Heterotrichea Stein, 1859 are found in a wide range of aquatic biotopes. Heterotrichs are characterized by their typically large body size, somatic kineties composed of dikinetids with postciliodesmata and a prominent oral apparatus composed of a paroral membrane and an adoral zone of membranelles [1, 2]. According to the latest classification [3], the class Heterotrichea contains ten families and about 58 genera. The genus Gruberia Kahl, 1932 has four valid species: G. binucleata Dragesco, 1960, G. lanceolata (Gruber, 1884) Kahl, 1932, G. nematodomorpha Lepsi, 1965 and G. uninucleata Kahl, 1932 [4]. Of these, only G. lanceolata has been investigated using modern methods while its congeners remain insufficiently described [4, 5].
The genus *Linostomella* Aescht in Foissner et al., 1999 is monotypic and is classified within the family Condylostomatidae Kahl in Doflein and Reichenow, 1929. It is characterized by its ovoid body shape, the anterior end of which is obliquely truncated by the wide buccal area, and a contractile vacuole near the posterior end [6, 7]. The type species, *L. vorticella*, was first reported by Ehrenberg [8] as *Bursaria vorticella*. However, neither its systematic position nor its evolutionary relationships with other taxa within the family Condylostomatidae have been clarified [9–19].

In the present study, populations of two poorly known heterotrich species, *Gruberia uninucleata* Kahl, 1932 and *Linostomella vorticella* (Ehrenberg, 1833), were isolated in Qingdao, China (Figure 1), giving the opportunity to investigate their taxonomy and phylogeny based on both morphological and molecular data.

**Results And Discussion**

**Family Gruberiidae Shazib et al., 2014**

**Genus Gruberia** Kahl, 1932

*Gruberia uninucleata* Kahl, 1932

**Synonyms**

1932 *Gruberia uninucleata* spec. n.—Kahl, Tierwelt Dtl 25: 441, [Figure 73] (original morphological description) (Present work: Table 2) [12].

1960 *Gruberia uninucleata* Kahl—Dragesco, Trav Biol Roscoff 12: 286, 287, [Figure 149] (brief redescription of living cell) (Present work: Table 2) [20].

1965 *Gruberia uninucleata* Kahl—Dragesco, Cah Biol Mar 6: 392, 393, [Figure 28] (incomplete morphological redescription) (Present work: Table 2) [21].

2002 *Gruberia uninucleata* Kahl, 1932—Dragesco, Linzer Biol Beitr 1561, 1562, [Figures 163–181 on page 1605-1607] (morphological redescription with incomplete infraciliature information) (Present work: Table 2) [22].

*Gruberia uninucleata* was originally reported by Kahl [12] and described as the type species of the genus. Although this organism has been repeatedly reported, an accurate definition and molecular data remain unavailable [12, 20–22]. We here present an improved diagnosis based on previous and
Improved diagnosis

Cell size about 200–800 × 30–50 μm in vivo. Body slender and slightly contractile, with conspicuously pointed caudal region. Oral area about 25%—45% of body length; 40–174 adoral membranelles; 23–75 fragments in paroral membrane. Pellicle with rod-shaped, dark-brownish cortical granules, forming 3–5 lines between adjacent ciliary rows; fusiform mitochondria densely arranged in regular lines between ciliary rows. About 16–40 somatic kineties including several shortened ventral kineties that form a suture near posterior end of body. Single sausage-shaped macronucleus. Marine habitat.

Voucher slides

Four voucher slides with protargol-stained specimens are deposited in the Laboratory of Protozoology, Ocean University of China (OUC) with registration numbers: CY2018122001–01, 02, 03, 04.

Morphological description of the Qingdao population (Figures 2–4, Table 1)

When fully extended, cell about 400–800 × 30–50 μm in vivo, on average about 560 × 39 μm, and 185–430 × 57–145 μm in protargol-stained specimens. Body flexible and slightly contractile (Figure 3F, G). Elongated body with length to width ratio about 10–18:1, anterior end beak-like, posterior part gradually narrows to a pointed end (Figures 2A, 3A–E, I). Length of oral area relative to body length highly variable, ranging from 25%—45% (Figures 2B, 3A–E). Adoral zone of membranelles conspicuous, cilia of membranelles 11–16 μm long. Oral groove slightly curved to right side, twisted as it enters oral opening, terminates at the visible cytopharynx (Figures 2H, I, 3A–G, 4B, D). Cilia of paroral membrane conspicuous, well-developed, 19–22 μm long in vivo (Figure 3K). Somatic cilia 5–7 μm long. Pellicle thick with rod-shaped, dark-brownish cortical granules (about 1.2 × 0.5 μm in size) embedded in cortex, forming 3–5 irregular lines between adjacent somatic kineties (Figures 2D, F, 3L, M). Mitochondria fusiform, about 2.0 × 0.7 μm in size, located underneath cortex forming three or four rows between adjacent ciliary rows, (Figures 2F, G, 3N).

Cytoplasm opaque at low magnification due to mass of small granules and food vacuoles (Figure 3A–G, J). Macronucleus sausage-shaped with an obvious depression (Figures 2E, 3O–Q, 4H). Micronucleus difficult to recognize either in vivo or in protargol preparations. Contractile vacuole absent.

Locomotion by gliding over substratum.
Adoral zone extends from apical end to main body, twisted in proximal region making a half-turn as it enters the oral opening (Figures 2H, I, 4B, D). About 76–174 adoral membranelles, each composed of one short and two long rows of basal bodies (Figures 2C, H, 4B, D). Paroral membrane fragmented into about 29–75 pieces and arranged along right side of adoral zone of membranelles, almost all fragments composed of two rows of kinetosomes except several anterior ones which comprise three rows; paroral membrane conspicuous, comprising bipartite anterior, fragmented main portion with each fragment composed of 2–5 pairs of kinetosomes, and posterior portion that is twisted but not fragmented (Figures 2C, H, I, 4B–E). Twenty-five to 37 somatic kineties composed of dikinetids, only one basal body of each dikinetid bears a cilium (Figures 2H, I, 3A, G). About 9–21 shortened somatic kineties, most of which originate from left margin of adoral zone of membranelles or oral cavity, remainder interspersed among bipolar kineties (Figures 2H, 4F). Several shortened kineties form a conspicuous suture on ventral side near posterior end of body (Figures 2I, 4I).

Comments on *Gruberia uninucleata*

The genus *Gruberia* was established by Kahl [12] with *G. uninucleata* as the type species. The morphology of *Gruberia* is similar to that of *Spirostomum* in having an elongated, slightly contractile body and a well-developed peristome region, although the body of *Gruberia* lacks spiraling or torsion [4, 23]. Based on this character, Kahl [12] transferred the well-known species *Spirostomum lanceolatum* to *Gruberia*. Seven nominal species of *Gruberia* have been reported: *G. aculeata* Ozaki & Yagi, 1941, *G. beninensis* Dragesco & Dragesco-Kernéis, 1986, *G. binucleata* Dragesco, 1960, *G. calkinsi* Beltran, 1933, *G. lanceolata* (Gruber, 1884) Kahl, 1932, *G. nematodomorpha* Lepsi, 1965, and *G. uninucleata* Kahl, 1932 [12, 16, 20, 24–27]. In their generic review, Chen et al. [4] synonymized *G. aculeata*, *G. beninensis* and *G. calkinsi* with *G. lanceolata*. We accept this decision and recognize only four valid species of *Gruberia*.

*Gruberia uninucleata* was originally discovered by Kahl [12] from an aquarium in Helgoland, Germany. According to the original description, this organism is ‘300–650 μm, slender and spindle-shaped body, pointed caudal region, somatic kineties 16–20, single ellipsoidal macronucleus’ (Table 2). The Qingdao population matches the original description in body shape and size, peristome length, macronucleus,
and habitat. The only significant difference is the number of somatic kineties (25–37 in Qingdao population vs. 16–20 in Helgoland population). As the number of somatic kineties was derived from living observation in the original description, it is likely that the author overlooked some shortened and marginal somatic kineties.

*Gruberia uninucleata* is a rare species. Apart from the original description, it has been reported only four times. Dragesco [20] isolated two populations from fine sands at Roscoff and Green Island, which corresponded closely to the Helgoland population except the Roscoff populations had a longer peristome and the Green Island population had a terminal contractile vacuole (Table 2). Based on our observations, we hypothesize that Dragesco [20] mistook a food vacuole for the contractile vacuole (Figure 3J). Subsequently, Dragesco [21] described a Port-Etienne population which is smaller (200 μm on average) than both the original and the Qingdao populations (Table 2). Chorik [28] reported a freshwater population of *G. uninucleata* which corresponds well with Kahl’s population (Table 2). However, we suspect that it is possibly a misidentification because it resembles *Blepharisma hyalinum* in terms of its body shape, macronucleus shape, a contractile vacuole at posterior end, and habitat [29]. Dragesco [22] supplied the most recent description of *G. uninucleata* based on living observations and infraciliature of another Roscoff population. This differs from the Qingdao population by having fewer somatic kineties (16–22 vs. 25–37 in Qingdao population) and fewer fragments in the main portion of the paroral membrane (23–29 vs. 29–75 in Qingdao population) (Table 2). We believe that this difference is probably a statistical error because only six individuals were counted by Dragesco [22].

In summary, the original population of Kahl [12] and that of Dragesco [22] both have about 20 somatic kineties. In contrast, the Qingdao population and that of Dragesco [21] both have about 40 somatic kineties. Based on the different numbers of somatic kineties, we believe that these populations represent two different species. However, further studies are needed, including molecular data, for the populations from France and Germany in order to test this hypothesis.

Family Condylostomatidae Kahl in Doflein & Reichenow, 1929

Genus *Linostomella* Aescht in Foissner et al., 1999
Linostomella vorticella (Ehrenberg, 1833) Aescht in Foissner et al., 1999

Synonyms
1833 Bursaria vorticella n. sp.—Ehrenberg, Abh dt Akad Wiss 237 (original description without illustration) (Present work: Table 3) [8].
1838 Bursaria vorticella Ehrenberg, 1833—Ehrenberg, Infusionstherien 326, 327 [Figure VI] (brief redescription) [9].
1841 Bursaria vorticella Ehrenberg—Dujardin, Librairie Encyclopédique de Roret 511 (without morphological description, only simple review of Ehrenberg’s works) [30].
1870 Condylostoma stagnale Wrześniowski, Z wiss Zool 20: 487–489 [Figure 20] (redescription of living morphology) (Present work: Table 3) [10].
1922 Condylostoma vorticella (Ehrenberg) Dujardin—Penard, Études Infusoirses 201, 202 [Figure 200] (morphological redescription based on living cell) (Present work: Table 3) [11].
1924 Condylostoma vorticella (Ehrenberg, 1833)—Fauré-Fremiet, Bull Biol Fr Belg 6: 136-139 [Figure 45] (redescription from life) (Present work: Table 3) [31].
1932 Condylostoma (Bursaria) vorticella (Ehrenberg, 1833)—Kahl, Tierwelt Dtl 25: 457 [Figures 12-14 on page 454, Figure 28 on page 458] (short revision with simple redescription) (Present work: Table 3) [12].
1933 Condylostoma vorticella (Ehrenberg) Dujardin 1841—Wang & Nie, Contr Biol Lab Sci Soc China 10: 45–48 [Figure 36] (redescription of morphology based on living cells) (Present work: Table 3) [19].
1967 Condylostoma vorticella Tuffraud, Protistologica 3: 381, 382 [Figure 7] (brief redescription) [13].
1974 Condylostoma vorticella (Ehrenberg)—Pätsch, Arb Inst landw Zool Bienenkd 1: 48, 49 [Figure 38] (brief redescription, including the infraciliature information) (Present work: Table 3) [14].
1978 Linostoma vorticella Ehrenberg—Jankowski, Tezisy Dokl zool Inst Akad Nauk SSSR, Jahr 39 (proposal for the establishment of genus Linostoma) [15].
1986 Condylostoma vorticella Ehrenberg,1833—Dragesco & Dragesco-Kernés, Faune Tropicale 391–393 [Figures A–D] (simple redescription including infraciliature information) (Present work: Table 3) [16].
1991 *Condylostoma vorticella* (Ehrenberg, 1838)—Packroff & Wilbert, Arch Protistenkd 140: 132-134 [Figure 7] (detailed morphological redescription from life and protargol-stained individuals) (Present work: Table 3) [17].

1992 *Linostoma vorticella* (Ehrenberg, 1833) Jankowski, 1978—Foissner et al., Informationsberichte des Bayer Landesamtes für Wasserwirtschaft 5/92: 390-393 [Figures 1-14] (diagnosis based on previous reports) (Present work: Table 3) [18].

1999 *Linostomella vorticella* (Ehrenberg, 1833) Aescht nov. nom. nov. comb.—Foissner et al., Informationsberichte des Bayerischen Landesamtes für Wasserwirtschaft 3/99: 655–661 [Figures 1–32] (improved diagnosis provided based on detailed morphological redescription) (Present work: Table 3) [7].

2007 *Linostomella vorticella* (Ehrenberg, 1838)—Alekperov et al., Protistology 5: 117, 118 [Figure 9, Plate 2D on page 114] (simple redescription) (Present work: Table 3) [6].

Prior to the current investigation, *Linostomella vorticella* has been found and reported numerous times, but some details of its morphology remain unknown. Based on both previous and present studies, an improved diagnosis is supplied.

**Improved diagnosis**

Body ovoid or ellipsoidal with anterior end obliquely truncated, cell size *in vivo* about 90–210 × 70–160 μm; buccal cavity conspicuous, length about 33%—60% of body length; 36–51 adoral membranelles; 26–51 somatic kineties; cortical granules colorless to dark-gray; single contractile vacuole posteriorly positioned with a long collecting canal; macronucleus moniliform with 2–15 nodules; freshwater and marine habitats.

**Voucher slides**

Three voucher slides with protargol-stained specimens are deposited in the Laboratory of Protozoology, Ocean University of China (OUC) with registration numbers: CY2019010501–01, 02, 03.

**Morphological description of the Qingdao population (Figures 5–7, Table 1)**

Cell size 135–205 × 70–110 μm *in vivo*, about 175 × 93 μm on average. Body ovoid with length to width ratio about 1.5–2.0:1 (Figures 5A, H, 6A–C). In general, anterior half wider than posterior half, apical end obliquely truncated, posterior end with a slight depression (Figures 5H, I, 6D, H). Buccal
cavity prominent, length about 35%—60% of body length with an oval glabrous protuberance visible in slightly squashed specimens (Figures 5B, H, 6I, M). Adoral zone of membranelles and paroral membrane prominent, cilia of adoral membranelles 20–30 μm long (Figures 5A, 6A, E). Somatic cilia 9–12 μm long. Pellicle soft and thin with numerous spherical, dark-gray cortical granules (about 0.9 μm in diameter) densely distributed between ciliary rows (Figures 5C, 6G).

Cytoplasm colorless, always filled with a mass of spheroidal particles and food vacuoles filled with algae (Figures 5A, 6J, K–N). Macronucleus moniliform with 5–12 nodules, located in middle portion of body (Figures 5A, E, H, 6N, O, 7A, C). Micronuclei inconspicuous, closely associated with macronuclear nodules (Figure 7G). Contractile vacuole in posterior region, varies in shape during diastolic process, with a collecting canal that extends to anterior region of body (Figures 5A, G, 6K–M). Locomotion by swimming while rotating about body axis.

Adoral zone composed of 36–51 membranelles, most of which consist of two rows of basal bodies of equal length; several adoral zone of membranelles in middle portion consist of three rows of basal bodies, third row with only two or three basal bodies (Figures 5D, F, 7E). Paroral membrane conspicuous, lies along right margin of buccal cavity (Figures 6A, E, 7B–D).

About 37–51 somatic kineties composed of dikinetids, only one basal body of each dikinetid bears a cilium (Figures 5D, E, 6F, 7H). About 11–18 ventral kineties are shortened since they originate below buccal cavity; all dorsal kineties extend complete length of cell (Figures 5D, E, 7A, B, F).

Comments on *Linostomella vorticella*

*Linostomella vorticella*, which is mainly found in freshwater, was originally reported as *Bursaria vorticella* by Ehrenberg [8]. It was subsequently named *Condylostoma vorticella* (Ehrenberg, 1833) and then *Linostoma vorticella* (Ehrenberg, 1833) [11, 15]. Aescht [32] reported that *Linostoma* Jankowski, 1978 is a homonym, thus she re-named it *Linostomella*. For nomenclatural purposes the genus and species names should be cited as *Linostomella Aescht in Foissner et al., 1999* and *Linostomella vorticella* (Ehrenberg, 1833) Aescht in Foissner et al., 1999, respectively [7].

*Linostomella vorticella* resembles *Condylostoma* in having an expansive oral region at the anterior end of the body and a conspicuous paroral membrane, therefore it was for a long time classified inthe
genus *Condylostoma*. The former can, however, be distinguished by the presence of a contractile vacuole (absent in *Condylostoma*), locomotion by free-swimming (vs. gliding in *Condylostoma*), lack of frontal membranelles (present in *Condylostoma*) and only one kinetosome of each dikinetid bears a cilium (both kinetosomes ciliated in *Condylostoma*) [33–36].

With reference to its general infraciliature, body shape and locomotion, *Linostomella* is also similar to the genus *Condylostomides*. However, the former can be separated from the latter by the lack of frontal membranelles (present in *Condylostomides*) [37].

As described above, *L. vorticella* was originally reported by Ehrenberg [8] under the name *Bursaria vorticella*. This description, however, was rather superficial and the failure to describe some important features (e.g. body size, macronuclear shape, etc.) made the subsequent re-identification of this organism difficult. According to the original and subsequent investigations this species should be recognizable by the following characters: (1) body shape spherical to ellipsoidal, posterior end rounded and anterior end always slightly truncated; (2) conspicuous oral cavity that occupies about half the body length; (3) macronucleus moniliform with nodules arranged in a horseshoe-shape or an oblique line; (4) contractile vacuole at the posterior end of the body with a long collecting canal (Table 3).

Gelei [38] reported an organism that resembles *L. vorticella* in all key characters except the number of somatic kineties (60–70 vs. 26–51 in populations of *L. vorticella*) (Table 3). We hypothesize that this population may represent a different species of *Linostomella*, however the description provided by Gelei [38] was only brief therefore its identity awaits further information. Dragesco [39] described an isolate collected from a freshwater pond in Mokolo, Cameroon, which has fewer adoral membranelles (19–22 vs. 36–51) than other populations of *L. vorticella* (Table 3). Foissner et al. [7] suspected that this population either represents a different species or was mis-observed. Alekperov et al. [6] reported the only marine population of *L. vorticella* but provided only a simple description (Table 3).

In general, the habitat is an important character for species circumscription, so further evidence is needed in order to verify the identity of this population.

*Phylogenetic analyses based on SSU rDNA sequences*
The two new SSU rDNA sequences obtained in this study were deposited in the GenBank database with lengths, G + C contents, and accession numbers as follows: *Gruberia uninucleata*, 1627bp, 46.22%, MN783327; *Linostomella vorticella*, 1683bp, 46.88%, MN783328. The ML and BI trees based on SSU rDNA data had nearly identical topologies, therefore only the ML tree is shown with support values from both analyses (Figure 8).

Seven sequences of *Gruberia* were included in the present analyses, i.e., the newly obtained sequence of *G. uninucleata* and six sequences obtained from the GenBank database. The seven sequences form a maximally supported clade (100% ML, 1.00 BI) that represents the family Gruberiidae in the SSU rDNA tree (Figure 8).

*Linostomella vorticella* and two other *Linostomella* sequences (LN869952, LN870136) cluster together with maximal support (100% ML, 1.00 BI), forming a sister-group to the *Condylostomides* assemblage (100% ML, 1.00 BI). The *Linostomella-Condylostomides* clade comprises one of the two sub-clades of the family Condylostomatidae; the other sub-clade contains the genera *Condylostoma*, *Chattonidium*, and *Condylostentor*.

Based on its fragmented paroral membrane, Shazib et al. [3] separated *Gruberia* from the family Spirostomidae and established for it the new family Gruberiidae. This assignment is supported by the present phylogenetic analyses, in which *Gruberia* is clearly divergent from the family Spirostomidae. All sequences of *Gruberia* form a clade that is the sister-group of the Stentoridae + Blepharismidae + Folliculinidae + Maristentoridae + Fabreidae clade (‘Clade I’ in Figure 8). This is congruent with the findings of previous studies [3–5, 40–42], and supports the scenario proposed by Luo *et al.* [41], which recognized that only species of ‘Clade I’ possess hypericin-like pigment granules. It is suggested that these pigment granules probably play important roles in the evolution of the class Heterotrichea, including the separation of *Gruberia* from ‘Clade I’ [40].

The new sequence of *L. vorticella* differs from the two unspecified sequences (LN869952, LN870136) by 14 and 9 nucleotides respectively. We suspect that these unspecified sequences may represent different species. The genus *Linostomella* is most closely related to *Condylostomides* in the SSU rDNA tree which is consistent with the morphological similarities of these two taxa and their placement in
the family Condylostomatidae [1]. The similarities include their habitat (freshwater), body shape (ellipsoidal), oral apparatus (conspicuous buccal cavity with adoral zone membrane on the left and paroral membrane on the right), presence of a contractile vacuole, and moniliform macronucleus [7, 37]. The monophyletic family Condylostomatidae comprises two clearly separated sub-clades, namely *Linostomella + Condylostomides* and *Condylostoma + Condylostentor + Chattonidium*. This division is probably related to the difference in habitat, members of the former clade inhabiting freshwaters whereas members of the latter clade are marine.

**Conclusions**

In the present paper we redescribe two poorly known heterotrich ciliates, *Gruberia uninucleata* and *Linostomella vorticella*, based on populations collected from Qingdao, China, using the integrative approach suggested by Warren *et al.* [43]. Moreover, the molecular data of these two species are provided for the first time. Improved diagnoses of these two species are supplied based on present and previous descriptions, and the phylogenetic relationships among related genera and species are discussed.

**Methods**

**Sample collection, morphological methods, and identification**

*Gruberia uninucleata* was collected from the sandy surface of a seawater aquarium in the Laboratory of Protozoology (N36°03′45″, E120°19′52″), Qingdao, China, on 20th December 2018; the water temperature was 24 °C and salinity was 30 PSU (Figure 1C). *Linostomella vorticella* was isolated from a freshwater pond in Baihuayuan Park (N36°03′53″, E120°20′22″), Qingdao, China, on 5th January 2019; the water temperature was 2 °C (Figure 1D).

Living cells were randomly selected from the original samples and observed at 100–1000× magnification using both bright field and differential interference contrast microscopy (Olympus BX53; Zeiss AXIO Imager. D2). The protargol staining method of Wilbert [44] was used to reveal the infraciliature. The protargol powder was made according to Pan *et al.* [45]. Hoechst 33342 solution was used to reveal the nuclear apparatus [46]. Counts, measurements, and drawings of stained specimens were made from photomicrographs (Nikon Y-IDT). Terminology and systematics are mainly according to Lynn [1] and Shazib *et al.* [3].
DNA extraction, PCR amplification, and sequencing

A single cell of each species was isolated from the original sample and washed five times with filtered habitat water to remove potential contaminants. Extraction of genomic DNA was performed using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. Q5® Hot Start high-fidelity DNA polymerase (NEB, Ipswich, MA) was used to amplify the SSU rDNA using universal eukaryotic primers 82F (5’-GAAACTGCGAATGGCTC–3’) and 18s-R (5’-TGATCCTTCTGAGGTTACACCTAC-3’) [47, 48]. Cycling parameters of touchdown PCR were as follows: 1 cycle of initial denaturation at 98 °C for 30 s, followed by 18 cycles of amplification (98 °C, 10 s; 69–51 °C touchdown, 30 s; 72 °C, 1 min), and another 18 cycles (98 °C, 10 s; 51 °C, 30 s; 72 °C, 1 min), with a final extension of 72 °C for 5 min. PCR products were checked using agarose gel and were sequenced in TSINGKE (Qingdao, China). Sequence fragments were assembled into contigs using Seqman (DNASTar).

Phylogenetic analyses

A total of 96 taxa were used for phylogenetic analyses, including the two newly sequenced species and 94 sequences obtained from the GenBank database (see Figure 8 for accession numbers). Five karyorelictean species were used as the outgroup. Sequences were aligned using MUSCLE on the web server GUIDANCE ((http://guidance.tau.ac.il/ver2/) with default parameters [49]. Ambiguously aligned regions were excluded before phylogenetic analyses using G-blocks version 0.91b [50, 51]. The final alignment with 1,431 characters was used to construct phylogenetic trees. Maximum likelihood (ML) analysis was carried out on the CIPRES Science Gateway [52] using RAxML-HPC2 on XSEDE v8.2.12 [53]. Bayesian inference (BI) analysis was performed with MrBayes version 3.2.6 on XSEDE [54, 55] of the CIPRES Science Gateway. GTR+ I+ G was selected as the best fitting evolutionary model by MrModeltest version 2.2 according to the Akaike Information Criterion (AIC) [56]. Markov chain Monte Carlo simulations were then run with two sets of four chains using the default settings. The chain length for the analysis was 10,000,000 generations with trees sampled every 100 generations. The first 10% of trees were discarded as burn-in. MEGA 5.2 [57] was used to visualize tree topology.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication

Not applicable.

Availability of data and materials
All data generated or analysed during this study are included in the published article.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
YC performed the experiments and drafted the manuscript; YL performed the phylogenetic section; MM, QZ, AW and XC checked all the data related and helped to improve the draft; WS supervised and organized to complete the work. All authors read and approved the final manuscript.

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Tables
Table 1. Morphometric data for Qingdao populations *Gruberia uninucleata* and *Linostomella vorticella*.
| Character                              | Species          | Min  | Max  | Mean | M   |
|---------------------------------------|------------------|------|------|------|-----|
| Body, length in vivo (µm)             | *G. uninucleata*| 400  | 800  | 560.0| 525.0|
|                                       | *L. vorticella*  | 135  | 205  | 175.0| 175.0|
| Body, width in vivo (µm)              | *G. uninucleata*| 30   | 50   | 39.3 | 35.0 |
|                                       | *L. vorticella*  | 70   | 110  | 93.2 | 95.0 |
| Body, length* (µm)                    | *G. uninucleata*| 185  | 430  | 325.1| 334.0|
|                                       | *L. vorticella*  | 150  | 269  | 205.4| 203.0|
| Body, width* (µm)                     | *G. uninucleata*| 57   | 145  | 87.1 | 86.0 |
|                                       | *L. vorticella*  | 111  | 204  | 154.7| 156.0|
| Oral area, length in vivo (µm)        | *G. uninucleata*| 145  | 295  | 200.7| 195.0|
|                                       | *L. vorticella*  | 55   | 110  | 80.0 | 85.0 |
| Oral area, length* (µm)               | *G. uninucleata*| 72   | 190  | 135.7| 140.5|
|                                       | *L. vorticella*  | 68   | 130  | 96.9 | 96.5 |
| Adoral membranelles, number           | *G. uninucleata*| 76   | 174  | 136.7| 141.0|
|                                       | *L. vorticella*  | 36   | 51   | 43.5 | 44.0 |
| Somatic kineties, number              | *G. uninucleata*| 25   | 37   | 32.4 | 32.5 |
|                                       | *L. vorticella*  | 37   | 51   | 42.4 | 42.5 |
| Shortened somatic kineties, number    | *G. uninucleata*| 9    | 21   | 14.4 | 14.5 |
|                                       | *L. vorticella*  | 11   | 18   | 13.5 | 13.0 |
| Fragments of paroral membrane, number | *G. uninucleata*| 29   | 75   | 56.7 | 55.5 |
|                                       | *L. vorticella*  | -    | -    | -    | -    |
| Ma nodules, number                    | *G. uninucleata*| 1    | 1    | 1.0  | 1.0  |
|                                       | *L. vorticella*  | 5    | 12   | 9.0  | 10.0 |
| Ma, length (µm)                       | *G. uninucleata*| 68   | 100  | 85.0 | 84.0 |
|                                       | *L. vorticella*  | 12   | 41   | 26.8 | 26.0 |
| Ma, width (µm)                        | *G. uninucleata*| 19   | 33   | 24.8 | 25.0 |
|                                       | *L. vorticella*  | 9    | 26   | 17.1 | 17.0 |

Abbreviations: CV, coefficient of variation in %; M, Median; Ma, macronucleus; Max, maximum; Mean,
arithmetic mean; Min, minimum; n, number of specimens; SD, standard deviation.

* Data based on protargol-stained specimens, † Macronuclear nodules were selected randomly in each individual, – Data not available.

Table 2. Morphometric comparison of *Gruberia uninucleata* populations and doubtful species reported under that name.

| Characters        | Body shape                                                                 | Body size       | Peristome length | Num AZM |
|-------------------|-----------------------------------------------------------------------------|-----------------|------------------|---------|
| Present work      | Slender and elongated lanceolate, flexible and slightly contractile          | 400-800 × 30-50 | 25% – 45% of body length | 76-1    |
| Kahl [12]         | Slender spindle-shaped                                                      | 300-650         | 25% – 33% of body length | –       |
| Dragesco [20]     | –                                                                           | 200-600         | –                | –       |
| Dragesco [21]     | Tail pointed                                                               | 200 in average  | ca. 27% of body length* | –       |
| Chorik [28]†      | –                                                                           | ca. 420         | ca. 35% of body length* | –       |
| Dragesco [22]     | Elongated body with a well-drawn beak and a pointed caudal region, slightly contractile | 250-600         | ca. 28% of body length* | 40-8    |

Abbreviations: AZM, adoral zone of membranelles; FPM, fragments of paroral membrane; SK, somatic kineties.
† Doubtful species, * Data from pictures, – Data not available.

Table 3. Morphometric comparison of *Linostomella vorticella* populations with significant data and doubtful species reported under that name.

| Character         | Body shape                                                                 | Body length |
|-------------------|-----------------------------------------------------------------------------|-------------|
| Present work      | ellipsoidal and variable, obliquely truncated at the anterior end, a depression at the posterior end | 135–205     |
| Ehrenberg [8]     | almost spherical body, large and oblique oral cavity in front               | –           |
| Wrzesniowski [10] | ovoid body with a broadly rounded rim                                        | 210         |
| Penard [11]       | ovoid body with broad back and truncated forward                            | 200         |
| Fauré-Fremiet [31]| globular, hemispherical or ovoid body with obliquely truncated anterior   | 100–125     |
| Kahl [12]         | bag-shaped, truncated in front                                              | 100–200     |
| Wang & Nie [19]   | ovoid body, large and evenly rounded toward the posterior extremity, truncated at the anterior end | 180         |
| Gelei [38]†       | –                                                                           | –           |
| Dragesco [39]†    | –                                                                           | 160         |
| Pätsch [14]       | –                                                                           | 140–170     |
| Dragesco & Dragesco-Kernéis [16] | oval body, rounded posteriorly                                          | 140–170     |
| Packroff & Wilbert [17] | bag-shaped, truncated in front, rounded at the back                   | 170–200     |
| Foissner *et al.* [7] | saccular to ellipsoidal, both ends broadly rounded, ventral anterior half obliquely truncated | 100–210     |
| Alekperov *et al.* [6] | ellipsoidal, rounded on anterior and posterior ends                          | 90–160      |

Abbreviations: AZM, adoral zone of membranelles; Ma, macronucleus; SK, somatic kineties.
† Doubtful species, a Ratio of oral length to body length, b Data from drawing or pictures, – Data not available.
Geographical location of Qingdao and photographs of the sampling sites. A, Portion of the map of China, showing location of Qingdao. B, The seawater aquarium from which Gruberia uninucleata was isolated. C, The freshwater pond from which Linostomella vorticella was isolated.
Figure 2

Gruberia uninucleata from life (A, B, D, F, G) and after protargol staining (C, E, H, I). A, Right-lateral view of a typical individual. B, Various individuals to show different body shapes and ratios of buccal length to body length. C, Pattern of the adoral zone of membranelles and paroral membrane, arrows show fragments with three rows of kinetosomes. D, Cortical granules distributed between the ciliary rows. E, Various macronuclear shapes, arrows mark the contracted regions. F, Schematic drawing of a cross-section of the cortex, arrow marks the cortical granules, arrowhead indicates the fusiform mitochondria. G, Fusiform mitochondria regularly arranged underneath cortex, arrowheads indicate the position of somatic kineties. H, Schematic drawing of the adoral membranelles and paroral membrane, arrows mark the anterior fragments consisting of two or three rows.
of kinetosomes, arrowheads show the shortened somatic kineties along the left margin of the adoral zone of membranelles. I, Ventral view to show the infraciliature and sausage-shape macronucleus. Scale bars = 135 μm (A), 110 μm (I).

Figure 3

Photomicrographs of Gruberia uninucleata from life (A–G, I–Q) and after DAPI staining (H).

A–G, General right-lateral views to show the different body shapes and ratios of buccal length to body length, arrows mark the cytopharynx, arrowheads indicate the macronucleus. H, DAPI-stained individual, to show the macronucleus. I, Right lateral view of anterior end of cell, arrow marks the rostral apex. J, Cytoplasm filled with many empty vacuoles. K, Details of paroral membrane, arrows mark the conspicuous cilia of the fragments of the paroral membrane. L, Cortical granules (arrows) arranged in 3–5 irregular
lines between adjacent somatic kineties. M, Longitudinal section of the cell to show the thick cortex, arrows mark the rod-shaped cortical granules. N, Fusiform mitochondria (arrows) under the cortex. O–Q, Various macronucleus shapes (arrows), arrowheads indicate the contracted region. Abbreviation: Ma, macronucleus. Scale bars = 150 μm (A–D), 100 μm (E, F), 75 μm (G, H).

Figure 4

Photomicrographs of Gruberia uninucleata after protargol staining. A, Right-lateral view of a typical specimen. B, Detail of oral apparatus, arrows mark the paroral membrane, arrowheads show the adoral zone of membranelles, double arrowheads indicate the cytopharynx. C, Enlargement of the anterior part of paroral membrane, arrows indicate the fragments consisting of three lines of kinetosomes, arrowheads mark the fragments consisting of two lines of kinetosomes. D, Enlargement of the posterior portion of paroral
membrane, arrows mark fragments composed of two rows of kinetosomes, arrowheads indicate the proximal region of the paroral membrane that is not fragmented. E, Ventral view of the adoral zone of membranelles and paroral membrane. F, Ventral view of the ciliary pattern, arrows mark the shortened kineties originating from left margin of adoral zone of membranelles or oral cavity, arrowheads mark the shortened kineties interspersed among bipolar kineties. G, Detail of dikinetids, arrows mark ciliated basal body. H, Macronucleus. I, Ventral view of posterior portion of infraciliature by the invertible function of Photoshop, asterisks mark a conspicuous suture. Abbreviation: Ma, macronucleus. Scale bars = 100 μm (A), 50 μm (B, I), 30 μm (C–E, H).
Linostomella vorticella from life (A–C, G–I) and after protargol staining (D–F). A, Ventral view of a typical individual, arrow marks the fully expanded contractile vacuole. B, Ventral view of a squashed cell, arrows indicate the oval glabrous protuberance in the buccal cavity. C, Cortical granules distributed between the ciliary rows. D, E, Ventral (D) and dorsal (E) views to show the ciliary pattern, oral ciliature and macronucleus. F, Schematic drawing of the adoral membranelles and paroral membrane. G, Sowing the diastolic process of the contractile vacuole. H, Various individuals to show the different body shapes, ratios of buccal length to body length and distribution of macronuclear nodules. I, Left-lateral views of different individuals, arrows mark the depression at posterior end of body. Scale bars = 95 μm (D, E), 45 μm (A).

Figure 6

Photomicrographs of Linostomella vorticella from life (A–N) and after DAPI staining (O). A–C, Various individuals to show the different body shapes and ratios of buccal length to body
length, arrows mark the prominent paroral membrane. D, H, Left-lateral views of different cells, arrows mark the depression at posterior end of body. E, Detail of oral area, arrows mark the adoral zone of membranelles, arrowheads show the paroral membrane. F, Detail of cilia, arrows denote each basal body bears a cilium. G, Tiny cortical granules densely distributed between ciliary rows. I, Detail of the glabrous protuberance in oral cavity. J, Food vacuoles with algae. K, Dorsal view of an individual full of food vacuoles, arrows mark the collecting canal. L, Contractile vacuole (arrows) near posterior end of body. M, Ventral view of a squashed cell, arrows indicate the glabrous protuberance, arrowheads mark different stages in the diastolic process of the contractile vacuole. N, Dorsal view of a cell, arrows mark the moniliform macronucleus. O, DAPI-stained individual to show the moniliform macronucleus. Abbreviation: Ma, macronucleus. Scale bars = 90 μm (B, H), 75 μm (C, L, O), 60 μm (A, D, K, M, N).
Figure 7

Photomicrographs of Linostomella vorticella after protargol staining. A, Ventral view of a typical individual. B–D, Photomicrographs modified with invertible function in Photoshop. B, Dorsal view of a cell, arrows mark the paroral membrane. C, Detail of the moniliform macronucleus and conspicuous oral ribs (arrows). D, Detail of oral apparatus, arrows indicate the adoral zone of membranelles, arrowheads show the paroral membrane. E, Detail of the adoral zone of membranelles, arrows denote some membranelles composed of three rows of basal bodies, one of which is very short. F, Detail of somatic kineties, arrows mark the shortened somatic kineties. G, Detail of the macronucleus and micronuclei (arrows). H, Detail of dikinetids, arrows indicate that only one basal body of each dikinetid bears a cilium. Abbreviation: Ma, macronucleus. Scale bars = 95 μm (A, B), 50 μm (C, D), 20 μm (G).
Maximum likelihood (ML) phylogenetic tree inferred from 18S rDNA sequences (91 heterotrichean and 5 karyorelictean taxa). The posterior probabilities from the Bayesian inference (BI) were mapped onto the ML tree. Asterisks indicate a mismatch in branching pattern between the ML and BI trees. The newly sequenced species in this study are shown in red font. The scale bar corresponds to 2 substitutions per 100 nucleotide positions.