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RESEARCH PAPER

# Taxonomy and systematics of a new pleurostomatid ciliate, *Pseudolitonotus spirelis* gen. et sp. n. (Protozoa, Ciliophora, Haptoria)

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## Abstract

Recent studies have revealed a high diversity of pleurostomatid ciliates in brackish habitats. Here, a novel species, *Pseudolitonotus spirelis* gen. et sp. n., isolated from a mangrove wetland of southern China, was investigated based on living observation, protargol staining, and molecular analyses. The new genus *Pseudolitonotus* gen. n. is characterized by the last left somatic kinety (LKn) being shortened and none of the right somatic kineties extending to the anterior end of the cell, thus distinguishing it from all known pleurostomatid genera. The type species, *Pseudolitonotus spirelis* sp. n., is characterized by the possession of two macronuclear nodules, 11–15 right and 7–9 left kineties, a single contractile vacuole subterminally located, extrusomes evenly spaced along the entire ventral margin and some forming an "apical group", two types of cortical granules, and the bottom of the oral slit invariably being twisted. *Litonotus gracilis* (Pan et al. Eur J Protistol 51:494–506, 2015) is believed to be another member of this new genus as its LKn and right somatic kineties are all shortened. Hence, a new combination, *Pseudolitonotus gracilis* (Pan et al., 2015) comb. n., is suggested and its diagnosis is improved. Molecular phylogenetic analyses based on SSU rDNA sequence data reveal that *Pseudolitonotus* gen. n. is monophyletic and groups with *Apolitonotus* (Pan et al. J Eukaryot Microbiol 67:252–262, 2020) of the family Protolitonotidae (Wu et al. Zool Scr 46:245–253, 2017). However, the familial assignment of this new genus is uncertain based on current data.

**Keywords** Biodiversity · *Litonotus gracilis* · Molecular phylogeny · New combination · New genus · New species

## Introduction

Ciliated protozoa (ciliates) are widely distributed in a range of habitats (Bai et al. 2020; Liu et al. 2021; Lynn 2008; Wu et al. 2021; Zhang et al. 2021). This includes marine ecosystems where they play key roles in ecological processes, such as the remineralization of nutrients, connecting the classic food chain and the microbial loop (Azam et al. 1983). Most of our knowledge of the diversity, systematics, and ecology of ciliates derives from studies of temperate ecosystems. Far less is known about ciliates from tropical and sub-tropical ecosystems. For instance, mangrove ciliates are relatively poorly studied (Hu et al. 2019). Thus, to understand mangrove ecosystems, we need to improve our appreciation of their ciliate biodiversity. Here, we take a step toward that end by examining one order that occurs in mangrove swamps and similar benthic habitats.

Ciliates of the order Pleurostomatida Schewiakoff, 1896 are characterized by their laterally flattened body, slit-like cytostome, densely ciliated right side, and sparsely ciliated left side with bristle-like cilia (Lynn 2008). To date, about 300 nominal species of pleurostomatids have been reported from marine, freshwater, and terrestrial habitats worldwide (Buddenbrock 1920; Carey 1992; Dragesco 1954, 1960, 1965; Kahl 1931; Song and Wilbert 1989; Song et al. 2009; Stokes 1893; Wu et al. 2021a, b). They are assigned to 13 genera and four families, mainly based on the ciliary patterns of both somatic and perioral kineties and features of

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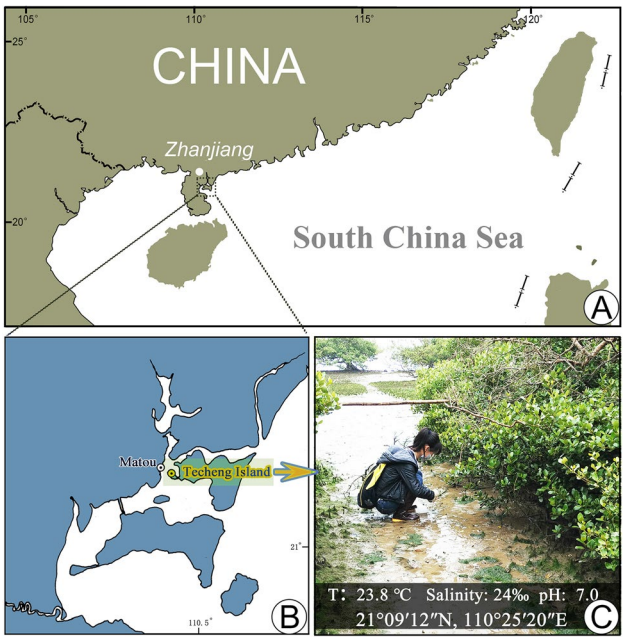


**Fig. 1** The sampling site. **A** Map of southern China with sampling area marked by the square. **B** Map of Techeng Island and surrounding area, with a yellow circle indicating the location of the sampling site. **C** A view of the sampling site

their extrusomes (Pan et al. 2020; Vďačný et al. 2015; Wu et al. 2017). Since the beginning of the twenty-first century, about 35 new species, three new genera and two new families have been reported from marine and brackish water habitats, including intertidal zones, mangrove wetlands, mariculture ponds, and estuaries (Chen et al. 2011; Hu et al. 2019; Lin et al. 2004, 2005a, b, 2007a, b, 2008; Pan et al. 2010, 2013, 2014, 2015, 2020; Song et al. 2009; Wu et al. 2013, 2014, 2015a, b, 2017, 2021a, b). Both single gene- and multiple gene-based phylogenetic analyses support the monophyly of the order Pleurostomatida (Pan et al. 2020; Vďačný et al. 2011, 2015, 2021a; Wu et al. 2017, 2021a, b; Zhang et al. 2012), which is consistent with morphological studies (Corliss 1979). However, there are several genera for which molecular data are lacking (*Heminotus, Opisthodon, Amphileptiscus* and *Apoamphileptus*) or for which only one SSU rDNA sequence is available (e.g., *Apolitonotus*, *Siroloxophyllum* and *Pseudoamphileptus*) in the GenBank database. Therefore, expanded sampling is needed to further explore the diversity of pleurostomatids using a range of methods (Warren et al. 2017).

In this study, a pleurostomatid was isolated from a mangrove wetland in the city of Zhanjiang, Guangdong Province, southern China (Fig. 1). After morphological and molecular studies and comparison with known species, this isolate could not be assigned to any known genus. Therefore, a new genus and new species, *Pseudolitonotus spirelis* gen. et sp. n., is suggested.

**ZooBank registration**

**This work:** urn:lsid:zoobank.org:pub:09138052-FC78-4BAF-96B4-1BC70E5B924F

***Pseudolitonotus* gen. n.:** urn:lsid:zoobank.org:act:E3D805BE-AD0B-4FE1-BA9D-1E5615C3DDB1

***Pseudolitonotus spirelis* sp. n.:** urn:lsid:zoobank.org:act:F1259A08-5894–4688-92B2-18EEFE6A8477

## Results

### Taxonomy

Class: Litostomatea Small and Lynn, 1981
Subclass: Haptoria Corliss, 1974
Order: Pleurostomatida Schewiakoff, 1896
Family: incertae familiae
Genus: *Pseudolitonotus* gen. n.

#### Diagnosis

Pleurostomatid in which the last left somatic kinety (LKn) is shortened and none of the right somatic kineties extend to the anterior end of the body.

#### Type species

*Pseudolitonotus spirelis* sp. n.

#### Etymology

The genus name is a composite of the Greek prefix "*Pseudo*" (not genuine; sham) and the generic name *Litonotus*, referring to its similarity to the well-known genus *Litonotus* Wrzesniowski, 1870 in terms of its body shape and ciliary pattern. Masculine gender.

#### Species assignable

*Pseudolitonotus spirelis* sp. n. and *Pseudolitonotus gracilis* (Pan et al., 2015) comb. n.

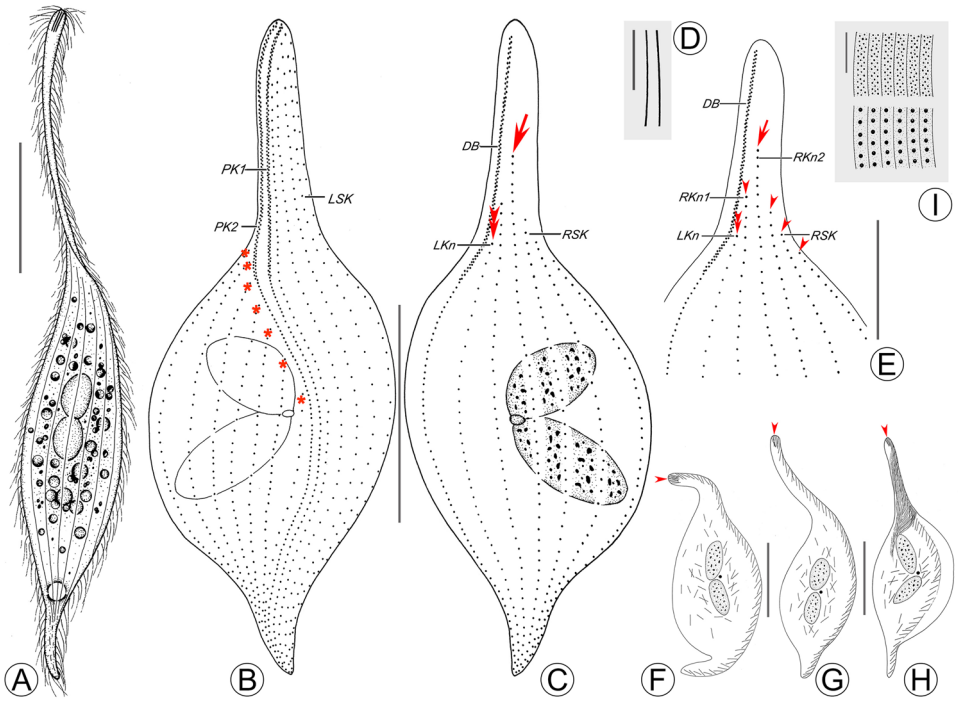


**Fig. 2** *Pseudolitonotus spirelis* gen. et sp. n. in vivo (**A**, **D**, **I**) and stained with protargol (**B**, **C**, **E–H**). **A** Right lateral view of a representative cell. **B**, **C** Ciliary patterns of right (**B**) and left (**C**) side of the holotype specimen, arrow indicates that the longest right somatic kinety does not extend to cell apex, asterisks mark the right somatic kineties in the leftmost region that are shortened along the perioral kineties, double arrowheads mark the shortening of the last left somatic kinety. **D** Extrusomes. **E** Ciliary pattern of anterior region of left side, arrowheads mark the right somatic kineties that do not extend to the cell apex, arrow shows the longest somatic kinety, double arrowheads point to the shortening of the last left somatic kinety. **F–H** Distribution of extrusomes, arrowheads show the "apical group". **I** Cortical granules. *DB* dorsal brush, *LKn* last left somatic kinety, *LSK* left somatic kinety, *PK1* perioral kinety 1, *PK2* perioral kinety 2, *RKn1* last right somatic kinety, *RKn2* penultimate right somatic kinety, *RSK* right somatic kinety. Scale bars: 50 μm in (**A–C**, **F–H**); 10 μm in (**D**, **E**, **I**)

### Remarks

This genus is distinguished from all known pleurostomatid genera by the shortened LKn and none of the right somatic kineties extending to the cell apex.

## *Pseudolitonotus spirelis* sp. n. (Figs. 2, 3; Table 1)

### Diagnosis

*Pseudolitonotus* about 160–350 μm in vivo; two macronuclear nodules; one micronucleus; 11–15 right and 7–9 left kineties; single contractile vacuole located subterminally; extrusomes bar-shaped, evenly spaced along entire ventral margin and some clustered together to form an "apical group" at anterior end of cell; bottom of oral slit invariably twisted; two types of cortical granules.

### Type locality and ecological features

A mangrove wetland on Techeng Island in the city of Zhanjiang, Guangdong Province, China (21°09′12″ N, 110°25′20″ E). Water temperature 23.8 °C, salinity 24‰, pH 7.0.

### Deposition of slides

A protargol slide with the holotype specimen circled in ink (registration no. WL2012040602-01A), and a second protargol slide with several paratype specimens (registration no. WL2012040602-01B), are deposited in the Laboratory of Protozoology, Ocean University of China (OUC), China.

### Etymology

The Latin adjective *spirel·is*, *-is*, -e ([m, f, n]; twist) refers to the bottom of "neck" (oral slit) invariably having a twist.

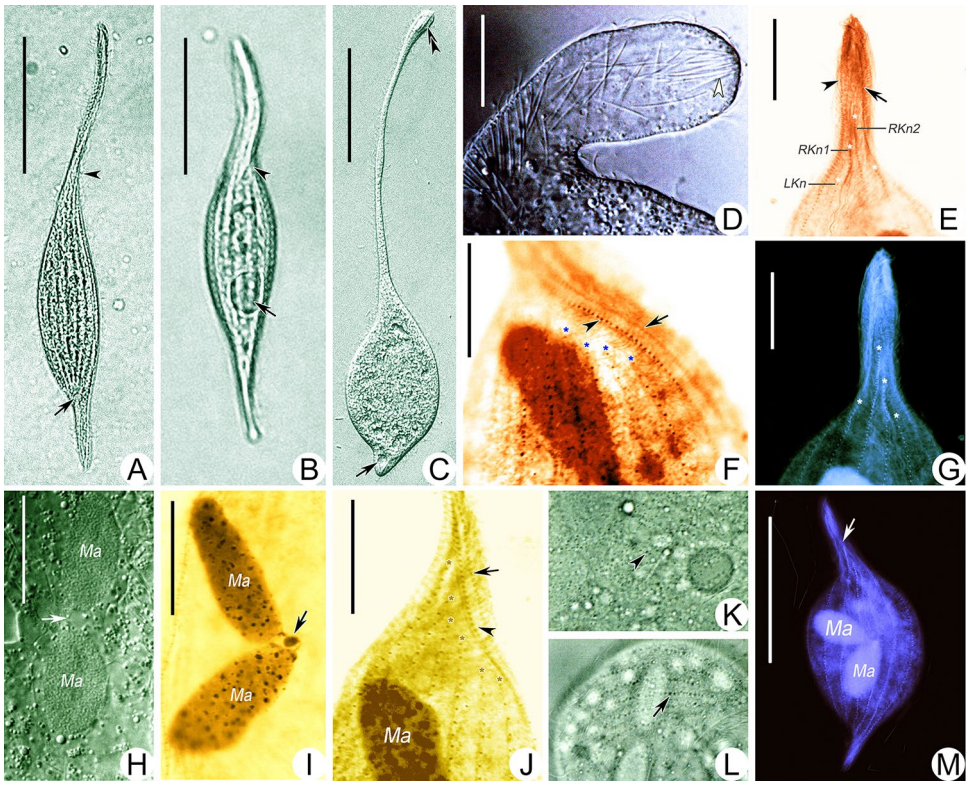


**Fig. 3** Photomicrographs of *Pseudolitonotus spirelis* gen. et sp. n. in vivo (**A–D**, **H**, **K**, **L**) and stained with protargol (**E–G***, **I**, **J**, **M***). **A** Right lateral view of a representative cell, arrowhead marks the twisted portion at the bottom of the oral area. **B**, **C** Contracted (**B**) and extended (**C**) individuals, arrowheads point to the twisted portion at the bottom of the oral area, arrows indicate the contractile vacuole, double arrowhead marks the "apical group" of extrusomes. **D** Anterior part of cell, arrowhead shows the "apical group" of extrusomes. **E** Left view of anterior region of cell of the holotype specimen, to show perioral kinety 2 (arrow), the dorsal brush kinety (arrowhead) and the shortened somatic kineties (asterisks). **G** Left view of anterior region of cell, asterisks mark the shortened somatic kineties. **H**, **I** Nuclear apparatus, arrow shows the micronucleus. **K**, **L** To show the smaller (arrowhead) and larger (arrow) cortical granules. **M** Ciliary pattern, arrow points to the longest somatic kinety (RKn2). *LKn* last left somatic kinety, *RKn1* last right somatic kinety, *RKn2* penultimate right somatic kinety. Scale bars: 100 µm in (**A–C**, **M**); 20 µm in (**D–J**). *Images **G** and **M** were false-colored to reveal structures (Adobe Photoshop CS6)

### Morphology and ciliary pattern

Body size about 160–350 µm × 30–45 µm in vivo. Body shape *Litonotus*-like, i.e., slender lanceolate, contractile, with sharply pointed posterior end and a long conspicuous neck-like region that is about half cell-length when fully extended (Figs. 2A, 3A, B). Bottom of oral area twisted to right side in all specimens ($n > 20$), conspicuously twisted when cell contracts (Figs. 2A, 3A, B), and can be detected in protargol-stained specimens (Fig. 3F, J). Two ovoidal macronuclear nodules, each about 20–25 µm × 10–15 µm in vivo, located in mid-body region (Figs. 2F–H, 3H, I). Single ovoidal micronucleus, located between macronuclear nodules, about 2–3 µm across (Fig. 3H, I). One contractile vacuole, about 15–20 µm in diameter, subterminally located (Figs. 2A, 3A–C). Extrusomes bar-shaped, about 15 µm long and 0.2 µm wide in vivo, densely and evenly spaced along entire ventral margin, some clustered together to form a conspicuous "apical group" at anterior end of cell (Fig. 2A, F–H) that can be detected with DIC microscopy (Fig. 3C, D). Pellicle thin with inconspicuous longitudinal furrows on right side within which ciliary rows are located (Fig. 3A). Two kinds of cortical granules: type 1 dot-like, ca. 0.2 µm across, grayish, irregularly scattered between ciliary rows (Figs. 2I, 3K); type 2 globular, ca. 1–1.5 µm across, grayish, regularly arranged in a single line between adjacent ciliary rows (Figs. 2I, 3L). Right side densely ciliated with cilia ca. 8 µm long; left side sparsely ciliated. Cytoplasm colorless to pale yellow, often with numerous refringent globules ca. 2 µm across and several food vacuoles 3–5 µm across that renders main part of body opaque (Fig. 3A, B). Locomotion by swimming or by gliding on substrate.

Ciliary pattern as shown in Figs. 2B, C, E, 3E–G, J, M. Excluding perioral kinety 2 (PK2), ten to 14 right somatic kineties (Figs. 2B, C, 3F, J) none of which extend to cell apex; penultimate right somatic kinety (RKn2) is the longest of them (Figs. 2C, E, 3E, G, M); leftmost region of RKn2 shortened along PK2; rightmost region of RKn2 shortened

**Table 1** Morphological characteristics of *Pseudolitonotus spirelis* gen. et sp. n. based on protargol impregnated specimens (all measurements in μm)

| Character | H | Min | Max | Mean | SD | *n* |
|---|---|---|---|---|---|---|
| Body length | 185 | 115 | 280 | 190.5 | 43.83 | 19 |
| Body width | 55 | 40 | 75 | 52.5 | 10.55 | 19 |
| Number of RK[a] | 13 | 11 | 15 | 12.2 | 1.31 | 19 |
| Number of LK[b] | 9 | 7 | 9 | 8.7 | 0.58 | 19 |
| Number of Ma | 2 | 2 | 2 | 2 | 0 | 19 |
| Length of Ma | 30 | 15 | 40 | 30.0 | 7.30 | 19 |
| Width of Ma | 10 | 8 | 20 | 12.4 | 3.50 | 19 |
| Number of DB | 61 | 46 | 78 | 59 | 8.53 | 19 |
| Distance to RKn2[c] | 35 | 25 | 60 | 36.5 | 9.26 | 19 |
| Length of Ex | 15 | 12 | 16 | 14.5 | 1.07 | 19 |
| Length of Na | 40 | 30 | 75 | 48.4 | 12.25 | 19 |

*DB* dorsal brush, *Ex* extrusomes, *H* holotype, *LK* left kineties, *Ma* macronuclear nodules, *Max* maximum, *Mean* arithmetic mean, *Min* minimum, *n* sample size, *Na* nematodesmata, *RK* right kineties, *RKn2* penultimate right kinety, *SD* standard deviation

[a]Perioral kinety 2 included

[b]Perioral kinety 1 and dorsal brush kinety included

[c]Distance from anterior end to the penultimate right kinety

along dorsal brush kinety (DB) (Figs. 2B, C, E, 3E–G, J). Left side with 7–9 ciliated kineties including perioral kinety 1 (PK1) and dorsal brush kinety (DB) which extends to about 40% of cell length and is composed of narrowly spaced dikinetids (Fig. 2B, C, E); last left somatic kinety (LKn) does not extend to cell apex and is shorter than last right somatic kinety (RKn1) (Figs. 2C, E, 3E, G).

Two perioral kineties along oral slit. Perioral kinety 1 left of oral slit, comprises dikinetids in anterior 40% and extends posteriorly as a row of monokinetids (Figs. 2B, 3F, J). Perioral kinety 2 right of oral slit, comprises regularly spaced dikinetids in anterior 40% and monokinetids in posterior 60% (Figs. 2B, 3F, J). Perioral kineties invariably twisted towards right side (Fig. 3F, J). Nematodesmata well-developed, all originating from kinetosomes of perioral kinety and extending into cytoplasm (Fig. 2H).

### SSU rDNA sequence and phylogenetic analyses

The SSU rDNA sequence of *Pseudolitonotus spirelis* sp. n. has been deposited in GenBank with accession number, length, and GC content as follows: MT653620, 1492 bp, 43.16%. The sequence identities of the SSU rDNA between the new species and its morphologically similar and closely related species were 91.0–99.9%, i.e., 1 to 134 nucleotide site differences (Figs. 4, 5).

The topologies of trees constructed using each of the two algorithms were almost identical, so only the maximum likelihood (ML) tree is shown (Fig. 6). In both analyses, the order Pleurostomatida is monophyletic. The family Protolitonotidae is divided into three clades. The first clade comprises three sequences of two *Protolitonotus* species (*Pr. magnus* and *Pr. longus*). The second clade, which contains *Apolitonotus lynni*, is a sister group to the genus *Pseudolitonotus* gen. n., which is represented by *Ps. spirelis* sp. n. and two populations of *Ps. gracilis* comb. n. The third clade, which consists of *Protolitonotus clampi* and an unidentified ciliate, is sister group to the genus *Kentrophyllum* with moderate to full support (ML/BI, 93/1.00). The other two families, Amphileptidae and Litonotidae, are sister groups and each is monophyletic.

## Discussion

### Comments on *Pseudolitonotus* gen. n.

Ciliary patterns of both the somatic and the perioral kineties are important characters for the classification of pleurostomatids (Foissner 1984; Foissner et al. 1995; Lynn 2008). Among the known genera of the order Pleurostomatida, the last left somatic kinety (LKn) extends to the anterior end of the cell whereas in *Pseudolitonotus* gen. n. the LKn is obviously shortened. Consequently, this new genus can be separated from other pleurostomatid genera.

The ciliary pattern on the right side of the cell is one of the most important characters for the identification of pleurostomatids at family and/or genus level (Lynn 2008; Vďačný et al. 2015; Wu et al. 2017). Hitherto, the order Pleurostomatida was divided into four families based mainly on the ciliary pattern of the right side, i.e., Amphileptidae (single-suture), Epiphyllidae (double-suture), Protolitonotidae (semi-suture), and Litonotidae (no suture) (Vďačný et al. 2015; Wu et al. 2017). The right somatic kineties of

| Alignment length 1454 positions | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Ps. spirelis* sp. n. MT653620 | * | 99.8% | 99.9% | 93.1% | 93.2% | 92.5% | 93.5% | 91.0% | 91.1% | 91.0% | 96.9% | 92.6% |
| 2 | *Ps. gracilis* KP010148 | 2 | * | 99.9% | 93.1% | 93.2% | 92.4% | 93.4% | 90.8% | 91.0% | 90.9% | 96.9% | 92.5% |
| 3 | *Ps. gracilis* KT222269 | 1 | 1 | * | 93.2% | 93.2% | 92.4% | 93.4% | 90.9% | 91.0% | 91.0% | 96.9% | 92.6% |
| 4 | *Li. paracygnus* GQ351698 | 102 | 102 | 101 | * | 99.9% | 95.1% | 97.3% | 91.6% | 91.7% | 91.8% | 93.4% | 92.8% |
| 5 | *Li. paracygnus* DQ190464 | 101 | 101 | 100 | 1 | * | 95.2% | 97.4% | 91.6% | 91.8% | 91.9% | 93.4% | 92.8% |
| 6 | *Li. duplastriatus* KP010149 | 111 | 113 | 112 | 72 | 71 | * | 96.1% | 90.9% | 91.0% | 91.6% | 92.2% | 92.0% |
| 7 | *Li. pictus* GQ351699 | 96 | 98 | 97 | 39 | 38 | 57 | * | 92.0% | 92.1% | 91.9% | 93.3% | 93.1% |
| 8 | *Pr. magnus* KP870179 | 134 | 136 | 135 | 125 | 124 | 135 | 119 | * | 99.8% | 95.2% | 91.0% | 90.6% |
| 9 | *Pr. magnus* KP870177 | 132 | 134 | 133 | 123 | 122 | 133 | 117 | 2 | * | 95.3% | 91.1% | 90.8% |
| 10 | *Pr. longus* KP870181 | 133 | 135 | 134 | 121 | 120 | 124 | 120 | 71 | 69 | * | 90.8% | 91.4% |
| 11 | *Ap. lynni* MK736944 | 45 | 45 | 46 | 98 | 97 | 115 | 99 | 134 | 132 | 136 | * | 92.0% |
| 12 | *Pr. clampi* MK736945 | 109 | 111 | 110 | 107 | 106 | 118 | 102 | 139 | 137 | 128 | 118 | * |

**Fig. 4** The sequence identities (upper right) and numbers of nucleotide differences (lower left) of SSU rDNA sequences between *Pseudolitonotus spirelis* gen. et sp. n. and morphologically similar and/or closely related species. The new species is in bold font


**Fig. 5** Unmatched sites from SSU rDNA sequence alignment of *Pseudolitonotus spirelis* gen. et sp. n. with morphologically similar and/or closely related species sequences included in the phylogenetic analyses. Numbers indicate the unmatched site positions. Missing sites are indicated by dashes (-) and matched sites are marked with dots (.)

*Pseudolitonotus* gen. n. do not extend to the anterior end of cell but instead are progressively shortened from the middle to both sides, thus distinguishing it from all known pleurostomatid genera. *Pseudolitonotus* gen. n. cannot to be assigned to any of the four pleurostomatid families based on their current diagnostic characters (Vďačný et al. 2015; Wu et al. 2017). However, we are hesitant to suggest that the shortening of the right somatic kineties and/or the LKn is a diagnostic character at family level. Therefore, we regard *Pseudolitonotus* gen. n. as incertae familiae at this time.

## Revision of *Litonotus gracilis* Pan et al., 2015

*Litonotus gracilis* was originally described by Pan et al. (2015) who also sequenced its SSU rRNA gene. In their phylogenetic analyses, two populations of *Litonotus gracilis* clustered with *Kentrophyllum* rather than with its congeners or other litonotids (Pan et al. 2015). Although Pan et al. (2015) noted that some right somatic kineties (RSKs) of *L. gracilis* are shortened along the oral slit in the typical *Litonotus* pattern, they overlooked that none of the RSKs extend to the apical region of the cell (see Fig. 2**G** in Pan et al. 2015). We re-examined the type specimens of *Litonotus gracilis* and discovered that all of the RSKs are shortened, either along the dorsal margin or along the perioral kineties (Fig. 7B–D). Furthermore, Pan et al. (2015) did not recognize that the LKn, does not extend to the cell apex (Fig. 7A, E, F). The patterns of both the RSKs and LKn in *L. gracilis* are diagnostic characters of *Pseudolitonotus* gen. n. In addition, the two populations of *Litonotus gracilis* group with *Pseudolitonotus spirelis* sp. n. in the SSU rDNA tree to form a well-supported clade (ML/BI, 93/1.00)

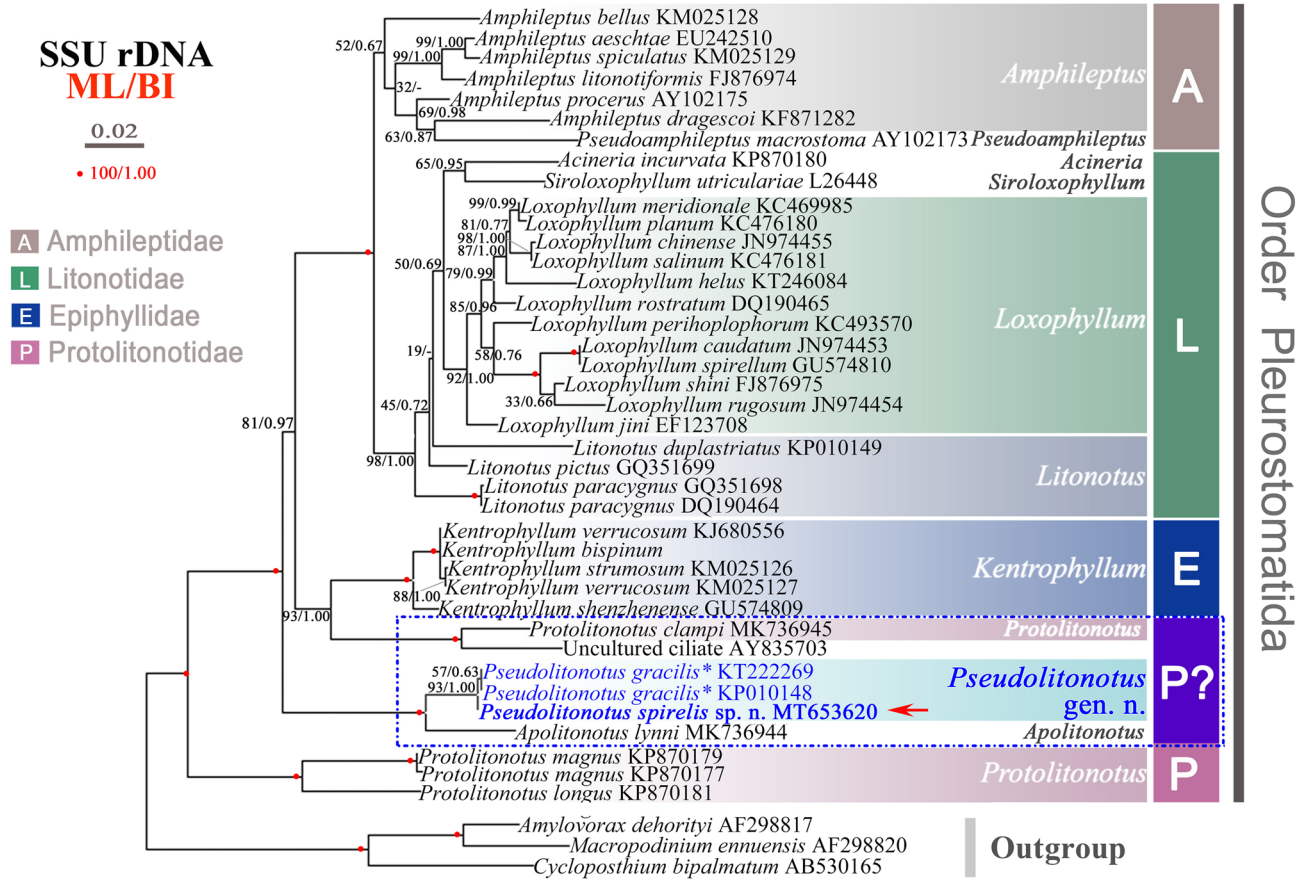


**Fig. 6** Maximum likelihood (ML) tree inferred from 42 SSU rDNA sequences of pleurostomatid and trichostomatid (outgroup) ciliates, revealing the phylogenetic position of *Pseudolitonotus spirelis* gen. et sp. n. (arrow). Bootstrap values of ML analysis and the posterior probabilities of Bayesian inference analysis (BI) are given at nodes. Dashes indicate incongruity between BI and ML trees. All branches are drawn to scale. GenBank accession numbers are given after names of species. Scale bar corresponds to two substitutions per 100 nucleotide positions. *Designated as *Litonotus gracilis* in Pan et al. (2015)

(Fig. 6). Hence, we suggest that *Litonotus gracilis* Pan et al., 2015 should be assigned to *Pseudolitonotus* gen. n. as a new combination, i.e., *Pseudolitonotus gracilis* (Pan et al., 2015) comb. n. (original combination: *Litonotus gracilis* Pan et al., 2015), and its diagnosis and ciliary pattern are improved based on original and current observations of the holotype and paratype specimens (Fig. 7).

## Improved diagnosis of *Pseudolitonotus gracilis* (Pan et al., 2015) comb. n.

Body about 200–350 μm in vivo, with conspicuous neck that is up to 50% of body length when fully extended; usually four macronuclear nodules; one contractile vacuole subterminally located; bar-shaped extrusomes arranged along oral silt; cortical granules arranged in honeycomb-like pattern; 5–9 left and 12–18 right kineties.

## Comments on *Pseudolitonotus spirelis* sp. n.

In terms of the body size and/or shape, seven pleurostomatid species resemble *Pseudolitonotus spirelis* sp. n., including: *Pseudolitonotus gracilis* (Pan et al., 2015) comb. n. (Fig. 7A, B); *Protolitonotus clampi* Pan et al., 2020 (Fig. 8C, D); *Apolitonotus lynni* Pan et al., 2020 (Fig. 8A, B); *Litonotus duplostriatus* (Maupas, 1883) Kahl, 1931 (Fig. 8G, H); *L. blattereri* Lin et al., 2008 (Fig. 8K, L); *L. gongi* Lin et al., 2009 (Fig. 8E, F); and *L. guae* Lin et al., 2009 (Fig. 8I, J) (Table 2). *Pseudolitonotus spirelis* sp. n. has two traits by which it can be distinguished from similar species: (1) extrusomes clustered together to form an "apical group" at the anterior end of the cell; and (2) the anterior part of the body twisted to the right at the bottom of the oral slit (Table 2). In addition, *Pseudolitonotus spirelis* sp. n. differs from *Ps. gracilis* (Pan et al., 2015) comb. n. by having fewer macronuclear nodules (2 vs. 4) and by the distribution patterns

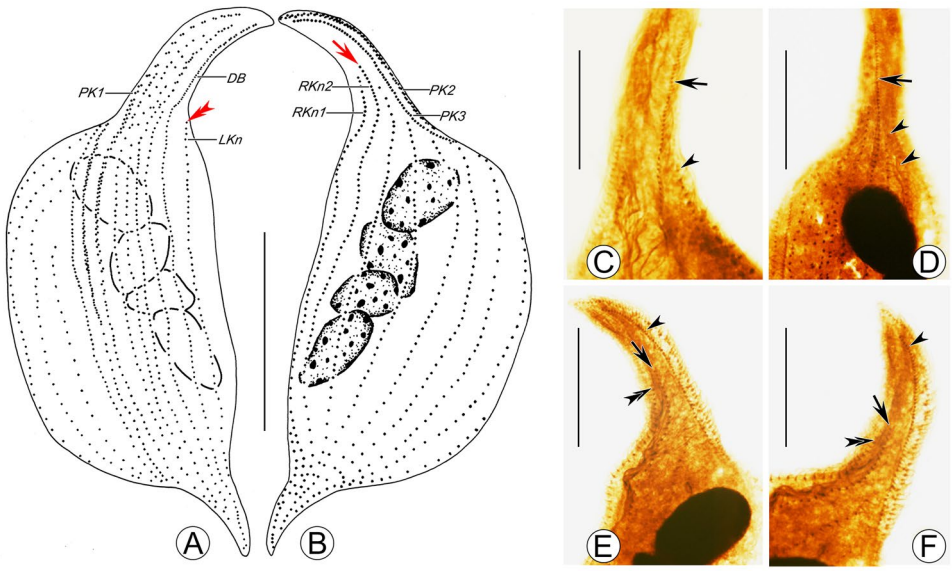


**Fig. 7** *Pseudolitonotus gracilis* (Pan et al., 2015) comb. n. stained with protargol (**A–F**). **A B** Ciliary patterns of right (**B**) and left (**A**) side of the holotype specimen, double arrowhead points to the shortened last left somatic kinety (LKn) and arrow marks the longest right somatic kinety (RKn2) not extending to cell apex. **C, D** Left view of anterior region of cell, to show the dorsal brush (arrow) and the last left somatic kinety (arrowhead). **E, F** Right view of anterior region of cell, to show the last right somatic kinety (double arrowhead), the penultimate right somatic kinety (arrow) and perioral kineties (arrowhead). *DB* dorsal brush, *LKn* last left somatic kinety, *PK1* perioral kinety 1, *PK2* perioral kinety 2, *PK3* perioral kinety 3, *RKn1* last right somatic kinety, *RKn2* penultimate right somatic kinety. Scale bars: 100 μm in (**A, B**); 10 μm in (**C–F**)

of extrusomes (along the entire ventral margin and with an "apical group" vs. along the oral slit only) (Pan et al. 2015).

### Comments on the phylogeny of *Pseudolitonotus* gen. n.

In the SSU rDNA tree, *Apolitonotus lynni* is the sister group to *Pseudolitonotus* gen. n. with full support (Fig. 6). This close relationship is unexpected considering the differences in their morphology. For example, *Pseudolitonotus* gen. n. can be clearly separated from *Apolitonotus* by a combination of: (1) the ciliary pattern on the right side (all right somatic kineties shortened vs. presence of several full-length right somatic kineties); (2) the presence (vs. absence) of extrusomes in the oral region; (3) the number of perioral kineties (2 vs. 3); and (4) the shortened (vs. not shortened) last left somatic kinety (Pan et al. 2020). The morphological characters of *Pseudolitonotus* gen. n. and *Apolitonotus* seem insufficient to reveal the phylogeny of either genus. Therefore, greater taxon sampling and more information on the species of *Apolitonotus* and *Pseudolitonotus* gen. n., including morphogenetic data and sequences of multiple gene markers, are needed to reveal their evolutionary relationships and to determine the family assignment of *Pseudolitonotus* gen. n.

## Materials and methods

### Sample collection and cultivation

*Pseudolitonotus spirelis* gen. et sp. n. was isolated from a mixture of water and rotting leaves collected on 06 April 2012 from a mangrove wetland (21° 09′ 12″ N, 110° 25′ 20″ E) on Techeng Island in the city of Zhanjiang, Guangdong Province, China (Fig. 1), when the water temperature was 23.8 °C, the salinity was 24‰, and the pH was 7.0. Ciliates were cultured at ca. 25 °C in Petri dishes containing about 20 ml of habitat water and two rice grains to facilitate the growth of bacteria as a food source for the ciliates.

### Sample observation and identification

Observations of isolated living cells were performed using bright field and differential interference contrast (DIC) microscopy (Nikon Eclipse 80*i*, Tokyo, Japan) at 100–1,000 × magnifications. Cells were protargol stained following the method of Wilbert (1975). Meristics and morphometrics were obtained from 19 stained specimens at a magnification of 1000 ×. Line diagrams of stained specimens were made with the help of a camera lucida at a magnification of 1250 ×. Terminology and classification followed that of Wu et al. (2017) and Vďačný et al. (2015).

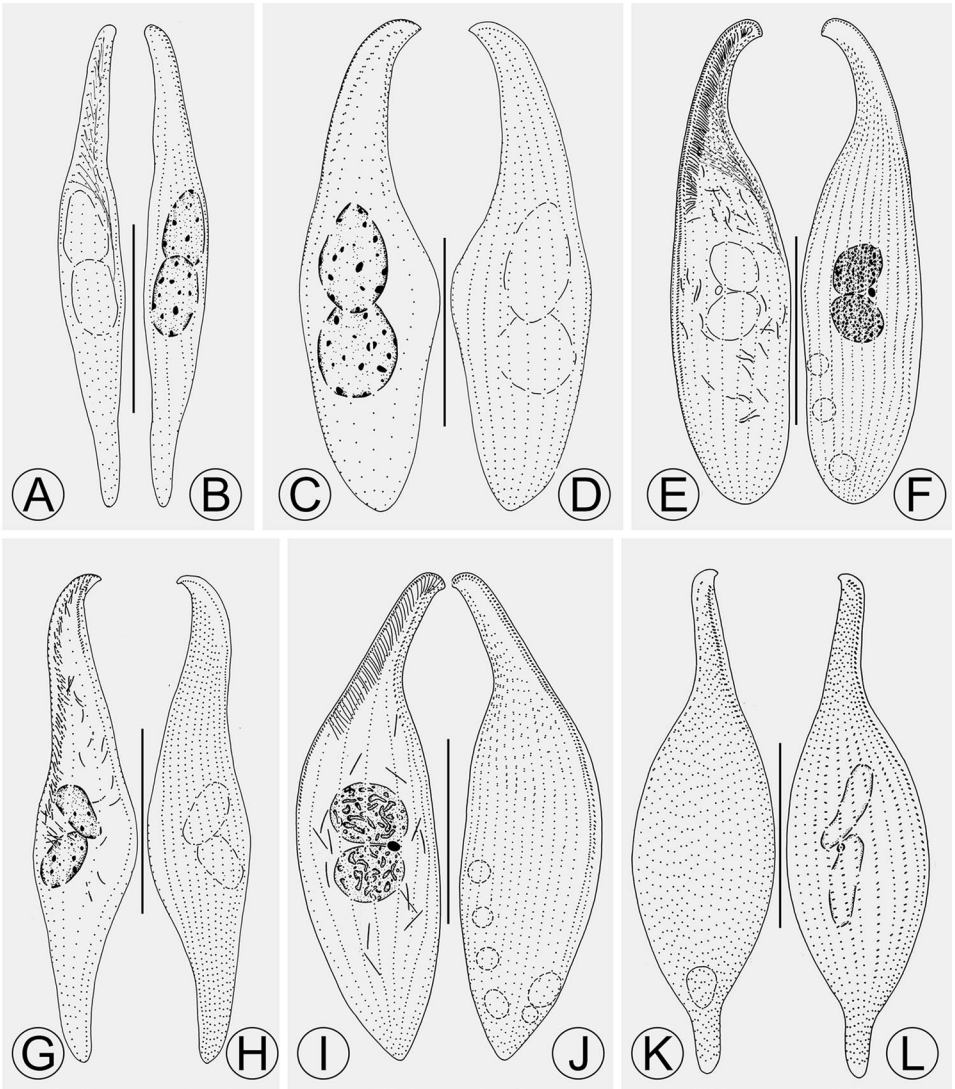


**Fig. 8** Morphology of species related and/or morphologically similar to *Pseudolitonotus spirelis* gen. et sp. n. **A, B** *Apolitonotus lynni*, from Pan et al. (2020). **C, D** *Protolitonotus clampi*, from Pan et al. (2020). **E, F** *Litonotus gongi*, from Lin et al. (2009). **G, H** *Litonotus duplostriatus*, from Pan et al. (2015). **I, J** *Litonotus guae*, from Lin et al. (2009). **K, L** *Litonotus blattereri*, from Lin et al. (2008). Scale bars: 50 μm in (**A–D**); 100 μm in (**E–L**)

## DNA extraction, PCR amplification and sequencing

Two cells were isolated from the raw cultures, rinsed five times with filtered habitat water (0.22 μm pore size) and then transferred into microfuge tube with ATL buffer. Genomic DNA extraction was performed with a DNeasy Blood & Tissue kit (Qiagen, Shanghai, China) according to the supplier's instructions. Gene amplification and gene sequencing were carried out following the methods described by Wu et al. (2013).

## Phylogenetic analyses

In addition to the new sequence of *Pseudolitonotus spirelis*, 38 SSU rDNA sequences of other pleurostomatids, including examples of all available genera within the order Pleurostomatida, were acquired from the GenBank database for the phylogenetic analyses (GenBank accession numbers are provided in Fig. 6). Three species of the order Trichostomatia, i.e., *Amylovorax dehorityi* AF298817, *Macropodinium ennuensis* AF298820 and *Cycloposthium bipalmatum* AB530165, were selected as outgroup taxa. Sequences were aligned using Clustal W implemented in Bioedit v. 7.2.6 (Hall 1999) with default parameters and edited and checked by eye to remove primer sequences and highly variable regions. The final alignment was used to construct phylogenetic trees included 1516 characters and 42 taxa.

Maximum likelihood (ML) analysis was conducted using RaxM-HPC2 V. 7.2.8 on XSEDE V. 8.1.11 with parameter settings as given by Stamatakis et al. (2008) via the CIPRES Portal V. 1.15 (http://www.phylo.org). The reliability of internal branches was estimated by bootstrapping with 1000 replicates. Bayesian inference (BI) analysis

**Table 2** Comparison of *Pseudolitonotus spirelis* gen. et sp. n. with morphologically similar species

| Species | BL[a] (μm) | RK[b] /LK[c] | n-MA[d] | pr-AP[e] | pr-T[f] | d-Ex[g]c | n & po-CV[h] | Data source |
|---|---|---|---|---|---|---|---|---|
| *Pseudolitonotus spirelis* | 260–350 | 11–15/7–9 | 2 | Yes | Yes | Entire ventral | 1, S | Present work |
| *Pseudolitonotus gracilis* | 200–450 | 12–18/5–9 | 4 | No | Yes | Oral slit | 1, S | Pan et al. (2015) |
| *Protolitonotus clampi* | 80–130 | 9–11/5 or 6 | 2 | No | No | Oral slit | 1, T | Pan et al. (2020) |
| *Apolitonotus lynni* | 100–180 | 5–7/4 or 5 | 2 | No | No | Scatter | 1, S | Pan et al. (2020) |
| *Litonotus duplostriatus* | 90–315 | 11–14/5 or 6 | 2 | No | No | Oral slit | 1, S | Pan et al. (2015) |
| *Litonotus blattereri* | 100–180 | 15–20/10–14 | 2 | No | No | Entire ventral | 1, T | Lin et al. (2008) |
| *Litonotus gongi* | 150–300 | 11–17/8–10 | 2 | No | No | Oral slit | 2–4, D | Lin et al. (2009) |
| *Litonotus guae* | 100–200 | 11–18/6–9 | 2 | No | No | Oral slit | 3–7, D & V | Lin et al. (2009) |

*AP* apical group, *BL* body length, *CV* contractile vacuoles, *d* distribution, *D* dorsal, *Ex* extrusomes, *LK* left kineties, *MA* macronuclear nodules, *n* number, *po* position, *pr* presence, *RK* right kineties, *S* subterminal, *T* terminal, *V* ventral

[a]Body length in vivo

[b]Perioral kineties 2 and/or 3 included

[c]Perioral kinety 1 and dorsal brush kinety included

[d]Number of macronuclear nodules

[e]Presence of apical group

[f]Presence of twist at the bottom of oral area

[g]Distribution of extrusomes

[h]Number and position of contractile vacuoles

was conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the GTR + G + I evolutionary mode indicated by MrModeltest v. 2.0 (Nylander 2004). The chain length of Markov chain Monte Carlo algorithm simulations was run for $10^6$ generations with trees sampled every 100 generations. The first 2500 generations (25%) were discarded as burn-in.

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**Author contributions** LW and XL conceived and planned the experiments. LW performed all the experiments and analyzed the phylogeny and wrote the manuscript with support from AW, XL and JL. XL supervised the project. All authors prepared the manuscript and approved the final version.

## Declarations

**Conflict of interest** Author Alan Warren is a member of the Editorial Board for Marine Life Science & Technology. He was not involved in the journal's review of, or decisions related to, this manuscript.

**Animal and human rights statement** We declare that all applicable international, national, and/or institutional guidelines for sampling, care, and experimental use of organisms for the study have been followed and all necessary approvals have been obtained.

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