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Diversity of Pleurostomatid Ciliates: Morphology, Taxonomy and Molecular Phylogeny of Freshwater Isolates Found in a Northern China Wetland, with a Description of Two New Species †

Gongaote Zhang [1,‡], Yongqiang Liu [1,‡], Hongbo Pan [2], Yujie Liu [1], Honggang Ma [1], Zhe Wang [1], Khaled A. S. Al-Rasheid [3], Weibo Song [1,*] and Hunter N. Hines [4,*]

1 Key Laboratory of Evolution & Marine Biodiversity (Ministry of Education), Institution of Evolution and Marine Biodiversity, Ocean University of China, Qingdao 266003, China; liuyongqiang@ouc.edu.cn (Y.L.); liuyujie@ouc.edu.cn (Y.L.)
2 Engineering Research Center of Environmental DNA and Ecological Water Health Assessment, Shanghai Ocean University, Shanghai 201306, China; hbpan@shou.edu.cn
3 Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia; krasheid@ksu.edu.sa
4 Harbor Branch Oceanographic Institute, Florida Atlantic University, Fort Pierce, FL 34946, USA
* Correspondence: wsong@ouc.edu.cn (W.S.); hunter.n.hines@gmail.com (H.N.H.)
† LSID[urn:lsid:zoobank.org:pub:E3991A92-A747-468B-9FAA-F670EA424CFC].
‡ These authors contributed equally to this work.

Abstract: Ciliates of the order Pleurostomatida play essential functions in microbial food webs from a variety of habitats and have been thought to possess a high level of diversity. Due to undersampling and often absent molecular data, the actual diversity and phylogenetic relationships within this group remain unclarified. To help address this deficiency, a survey of freshwater pleurostomatid ciliates was undertaken in Lake Weishan Wetland, northern China. Here, two new *Amphileptus* species, *Amphileptus sinicus* sp. nov. and *Amphileptus piscinarius* sp. nov., were investigated using modern morphological and molecular techniques. *Amphileptus sinicus* sp. nov. is characterized by possessing a comparatively large cell size of 330–490 μm, contractile vacuoles on both ventral and dorsal margins, and 8–10 left and 42–61 right kineties. *Amphileptus piscinarius* sp. nov. is characterized by possessing a cell size of 140–210 μm, a large distinctly developed apical extrusome group, 3–4 contractile vacuoles on the ventral margin, and 6–8 left and 24–28 right kineties. Phylogenetic results based on the 18S rRNA gene data of these two species group them with other congeners, with these data suggesting the genus *Amphileptus* is paraphyletic.

Keywords: Pleurostomatida; diversity; *Amphileptus*; taxonomy; 18S rRNA gene

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1. Introduction

The ciliated protists are a highly differentiated group of single-celled eukaryotes which exhibit a diversity of morphological features and play key roles in various ecosystems. Found within aquatic habitats are several iconic ciliates, for example, freshwater *Stentor* spp. are found free-swimming with frequent temporary attachment to substrates; the freshwater *Loxodes* spp. are typically found within the upper layers of sediments or above this layer in the water column as they are sensitive to oxygen gradients and light and move accordingly, thus being well suited for exploiting depleted and anoxic environments; euplotids feed on bacteria, microalgae and small protists and are commonly found in marine, freshwater and terrestrial habitats; *Amphileptus* spp. are widely distributed in freshwater, marine and brackish water habitats [1–12]. The order Pleurostomatida Schewiakoff, 1896 is a common and diverse group of raptorial feeders with developed extrusomes that show a marked preference for more sedentary prey, such as peritrichs and rotifers [13–17]. Pleurostomatida can be distinctly separated from other groups as being a bilaterally compressed cell; with

an oral slit located along the ventral margin, with dorsal bristles on the left side [16,18,19]. Although this group has a long history of study, the taxonomy of this group is hindered due to limitations of earlier studies: (1) some features (e.g., contractile vacuoles, extrusomes) being described only from live observation (potentially leading to many synonyms). (2) There are no molecular datasets available for the former morphological descriptions. (3) The abundance of pleurostomatids detected in samples is often low. Thus, detailed morphology-based classification [18,20,21] and molecular data need to be explored and re-investigated [22,23]. Moreover, in the past few years, many studies have revealed that pleurostomatids exhibit high biodiversity in marine and brackish waters, and more species may be yet to be discovered [24–31].

Recent molecular phylogenetic analyses have divided pleurostomatids into five families, the Amphileptidae Bütschli, 1889, the Litonotidae Kent, 1882, the Epiphyllidae Vd'ačný et al., 2015, the Paralitonotidae Zhang et al., 2022 and the Protolitonotidae Wu et al., 2017 [32–34]. According to previous studies, the amphileptids are represented by five genera: *Amphileptus* Ehrenberg, 1830; *Apoamphileptus* Lin & Song, 2004; *Amphileptiscus* Song & Bradbury, 1998; *Pseudoamphileptus* Foissner, 1983; *Opisthodon* Stein, 1859. *Amphileptus* is the largest and oldest genus in the family Amphileptidae with a global distribution in a wide variety of habitats, usually free-swimming and typically gliding on the substrate [15,20,30,35–40]. The species in *Amphileptus* might prefer to live in the vicinity of aquatic plants or eutrophic sites, and the abundance of these species recorded from samples collected in open water bodies is usually lower than in aquaculture water bodies. The distinctive characters of *Amphileptus* species are (1) having a right anterior suture; (2) no postoral suture; (3) no perioral kinety running along both sides of the oral slit; (4) absence of a "spoon" shaped apex at the anterior end; (5) absence of extrusomes along the dorsal margins [41–45]. Despite recent interest in the family Amphileptidae, only two of five genera (*Amphileptus* and *Pseudoamphileptus*) currently have molecular information, resulting in the ambiguous phylogenetic positioning of the remaining three genera. To date, the genus *Amphileptus* contains approximately 50 species, with only about a third of these having corresponding molecular information. Previous studies based largely on morphology suggested monophyly for this group, and indeed species in this genus display a high degree of similarity, however, recent molecular work suggests a non-monophyly for the genus *Amphileptus* [37], with the sequences presented here not disputing those findings. Based on the recent molecular work and our present work [37,39], *Amphileptus* are divided into six subclades in the phylogenetic tree, and *A. qingdaoensis* is grouped with two *Pseudoamphileptus* species which causes *Amphileptus* to currently be labeled paraphyletic. Due to the lack of molecular information in amphileptids, paraphyly of *Amphileptus* is likely to be confirmed with the addition of further molecular data [46].

Here, we provide data on *Amphileptus sinicus* sp. nov. and *Amphileptus piscinarius* sp. nov. that were isolated from aquaculture ponds surrounding the freshwater Lake Weishan in northern China. Their morphology and molecular sequences are provided to examine the diversity and phylogeny of pleurostomatids.

2. Materials and Methods

2.1. Sample Collection (Figure 1)

Amphileptus sinicus sp. nov. was collected from a fish farming pond in Lake Weishan Wetland, northern China (N 34°46′19.85″, E 117°09′45.93″) on 15 March 2021 with a water temperature of 13 °C and salinity of 0‰ at the time of sampling. *Amphileptus piscinarius* sp. nov. was collected from a separate fish farming pond in Lake Weishan Wetland, northern China (N 34°46′11.16″, E 117°09′59.04″) on 4 April 2021 with a water temperature of 14 °C and salinity of 0‰ at the time of sampling. The water temperature was measured with a thermometer (Shuniu, China, B-016110) and salinity with a YSI (Professional Plus, Yellow Springs, OH, USA).

Amphileptus sinicus sp. nov. was collected directly from the sampling sites using pipettes (maximum volume ~50 mL); *Amphileptus piscinarius* sp. nov. was collected using a

plankton net (mesh size 20 µm). Wide mouthed plastic sampling containers with a volume of 500 mL were also used. After collection, samples were transferred into Petri dishes and immediately investigated.

Figure 1. Sampling locations and images of sampling sites in Lake Weishan Wetland, China. (**A**) map of a portion of China, Lake Weishan Wetland (red circle), and typical habitat in wetland, Lake Weishan Wetland; (**B**) sampling site of *Amphileptus sinicus* sp. nov.; (**C**) sampling site of *Amphileptus piscinarius* sp. nov. The map of this part of China was downloaded from: http://www.tianditu.gov.cn.

2.2. Observation and Identification

Live cells were observed by bright field and differential interference contrast microscopy at 100–1000× magnification [47] using Olympus equipment and software, i.e., a BX53 microscope, a DP74 camera and cellSens software. The protargol staining method of [48] was used to reveal the ciliary pattern and nuclear apparatus. Drawings of live specimens were based on photomicrographs and direct observations (hand-drawn with the help of a tablet device). Drawings of stained specimens were made with the aid of a drawing device (hand-drawn with the help of a tablet device). The drawing program used was "Huashijie Pro". Terminology and systematics are mainly according to [16,34,41].

2.3. DNA Extraction, PCR Amplification and Gene Sequencing

For each species, a single cell was isolated from the raw samples and washed five times with filtered habitat water (0.22 µm pore filters) to avoid contamination. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was performed with the *ApexHF* HS DNA Polymerase FS Master Mix (Accurate Biotechnology Hunan Co., Ltd, Changsha, China). The universal eukaryotic primers: (1) EukA (5′-AAYCTGGTTGATYYTGCCAG-3′), 900R (5′-ACTAGGACGGTATCTGATCG-3′), 900F (5′-CGATAGATACCGTCCTAGT-3′) and EukB (5′-CYGCAGGTTCACCTACRG-3′) for *Amphileptus sinicus* sp. nov. (2) 18S-11F-Karyo

(5′-GCCAGTAGTSATATGCTTGTCT-3′) and EukB (see above) for *Amphileptus piscinarius* sp. nov. [49]. PCR programs were performed as follows: 1 cycle of initial denaturation at 94 °C, followed by 30 cycles of amplification (98 °C, 10 s; 55 °C 10 s; 72 °C 5 s/kb). The PCR products were sequenced bidirectionally using the Sanger method by Qingdao WeiLaibio Technology Co., Ltd. (Qingdao, China). Sequencing fragments were assembled into contigs using SeqMan ver. 7.1 (DNASTAR) and the final partial 18S rRNA gene sequences were edited in BioEdit ver. 5.0.6 [50].

2.4. Phylogenetic Analyses

Along with the two newly obtained sequences, another 73 sequences were downloaded from the GenBank database, including 64 pleurostomatid ciliates and 11 other free-living litostomateans (outgroup) that were used for the phylogenetic analyses (for accession numbers, see Figure 2).

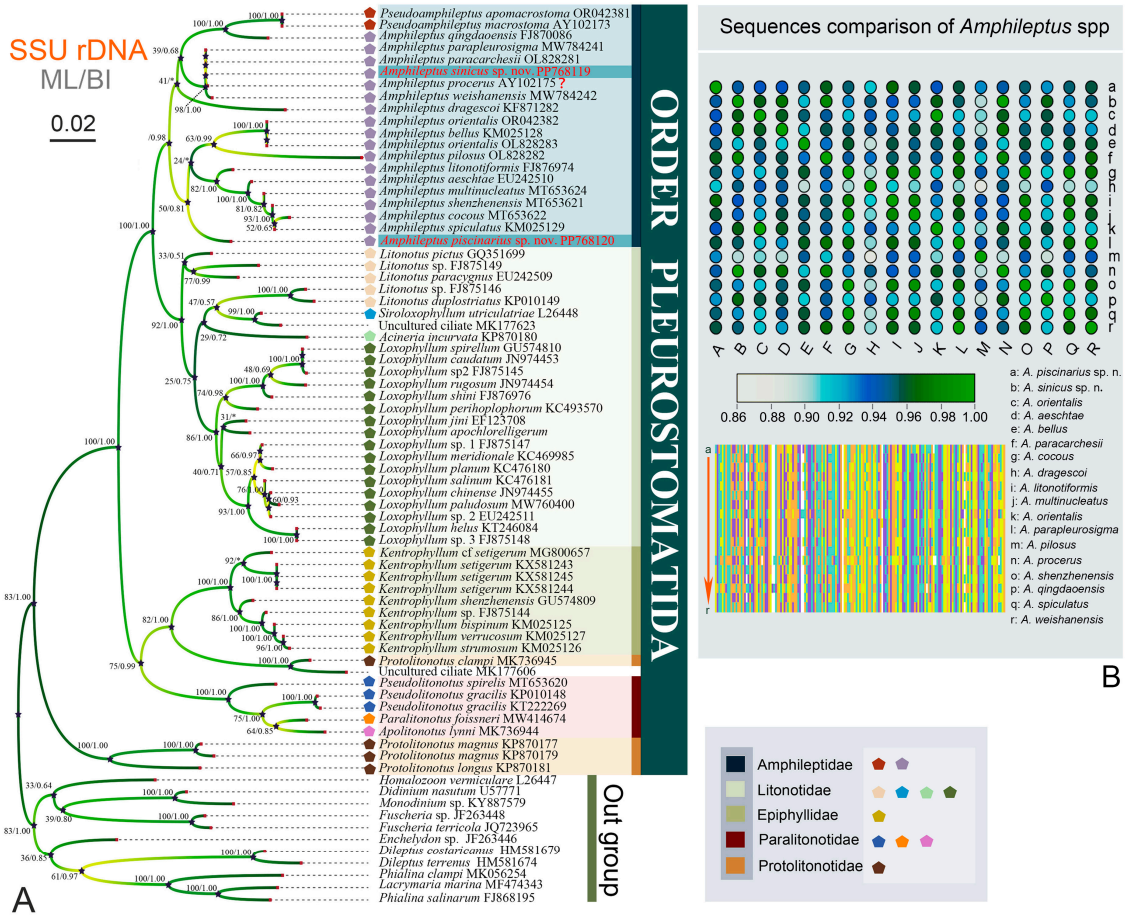

Figure 2. (**A**) Phylogenetic tree based on the 18S rRNA gene sequences, showing the systematic positions of *Amphileptus sinicus* sp. nov. and *Amphileptus piscinarius* sp. nov., denoted in red. Bootstrap values for maximum likelihood (ML) and posterior probabilities for Bayesian inference (BI) were mapped onto the best-scoring ML tree. The scale bar denotes one substitution per one hundred nucleotide positions. Clades with a different topology in the BI tree are indicated by an asterisk (*); (**B**) sequence comparison among *Amphileptus* species. The upper right diagram shows the similarities of species, with white to blue indicating the similarities from 0.86 to 1.00; the lower right diagram indicates the sites of nucleotide differences.

The 75 total sequences were aligned by MAFFT ver. 7.450 [51]. Ambiguously aligned regions were trimmed in Gblocks ver. 0.91b [52,53]. The final 18S rRNA gene sequence alignments comprising 1618 characters, including 438 variable and 344 parsimony-informative sites, were used for constructing phylogenetic trees using maximum likelihood (ML) and

Bayesian inference (BI) analyses. ML analyses were carried out with RAxML-HPC2 [54] on XSEDE ver. 8.2.12 on the CIPRES Science Gateway [55] under the GTRGAMMA model and with 1000 rapid bootstrap replicates. Bayesian inference analyses were conducted using MrBayes ver. 3.2.7 [56] under Akaike information criterion (AIC). Bayesian analyses were run for 10 million generations with a sampling frequency of 100. The first 10,000 trees were discarded as burn-in. MEGA ver. 7 was used to display the tree topologies [57].

3. Results

3.1. Taxonomy

Amphileptus species usually exhibit a similar cell shape, with the detailed morphological descriptions as below.

Class Litostomatea Small & Lynn, 1981
Subclass Haptoria Corliss, 1974
Order Pleurostomatida Schewiakoff, 1896
Family Amphileptidae Bütschli, 1889
Genus *Amphileptus* Ehrenberg, 1830
Amphileptus sinicus sp. nov. (Figures 3 and 4; Table 1)

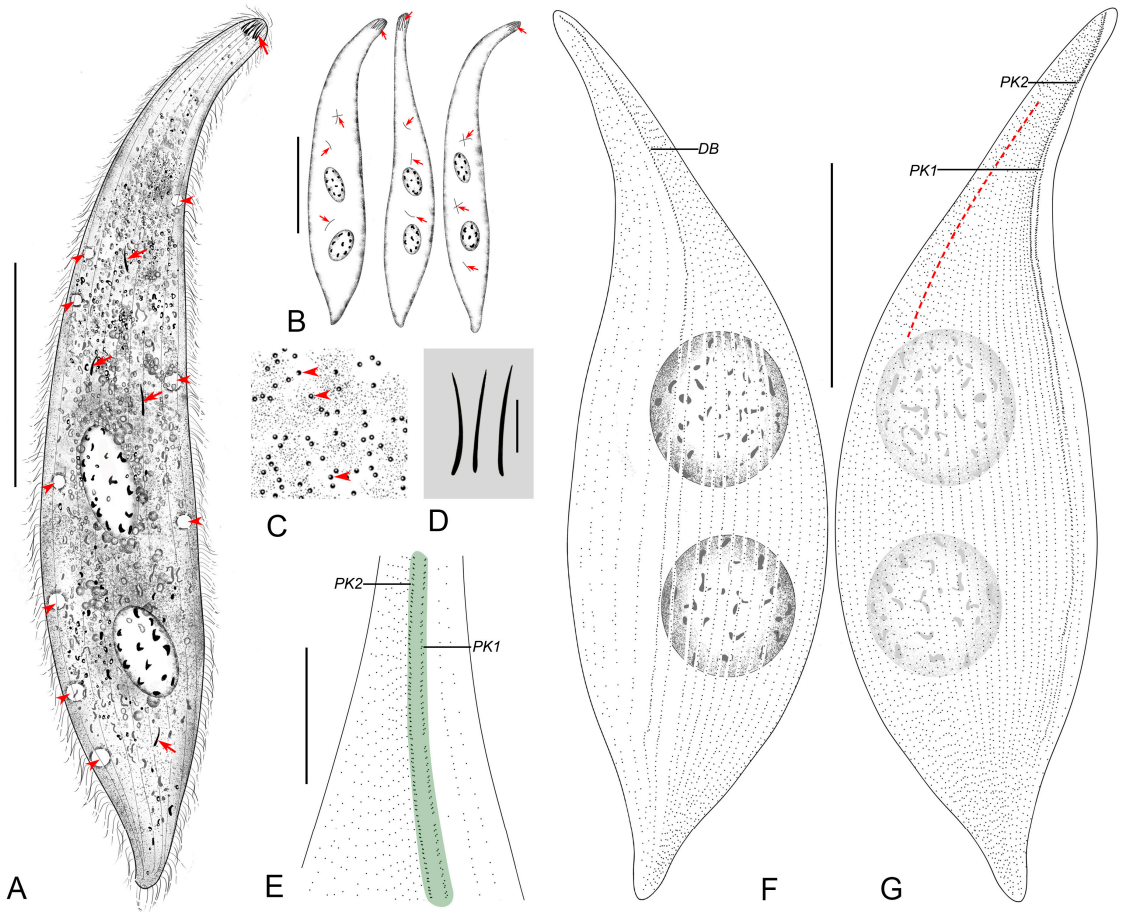

Figure 3. *Amphileptus sinicus* sp. nov., drawings of a living cell (**A**–**D**) and after protargol staining (**E**–**G**). (**A**) Left view of a representative individual, arrows indicate the extrusomes, arrowheads denote the contractile vacuoles on the ventral and dorsal margins; (**B**) shape variants, arrows indicate the extrusomes; (**C**) dot-like cortical granules (arrowheads); (**D**) narrowly cuneate extrusomes; (**E**) details of the oral ciliary pattern, green-shaded area shows the oral slit; (**F**) ciliary pattern of the dorsal side of the holotype specimen; (**G**) ciliary pattern of the ventro-lateral side of the holotype specimen, dashed line marks the right anterior suture. Abbreviations: DB, dorsal brush; PK1, perioral kinety 1; PK2, perioral kinety 2. Scale bars: 100 μm in (**A**,**B**,**F**,**G**), 5 μm in (**D**), 50 μm in (**E**).

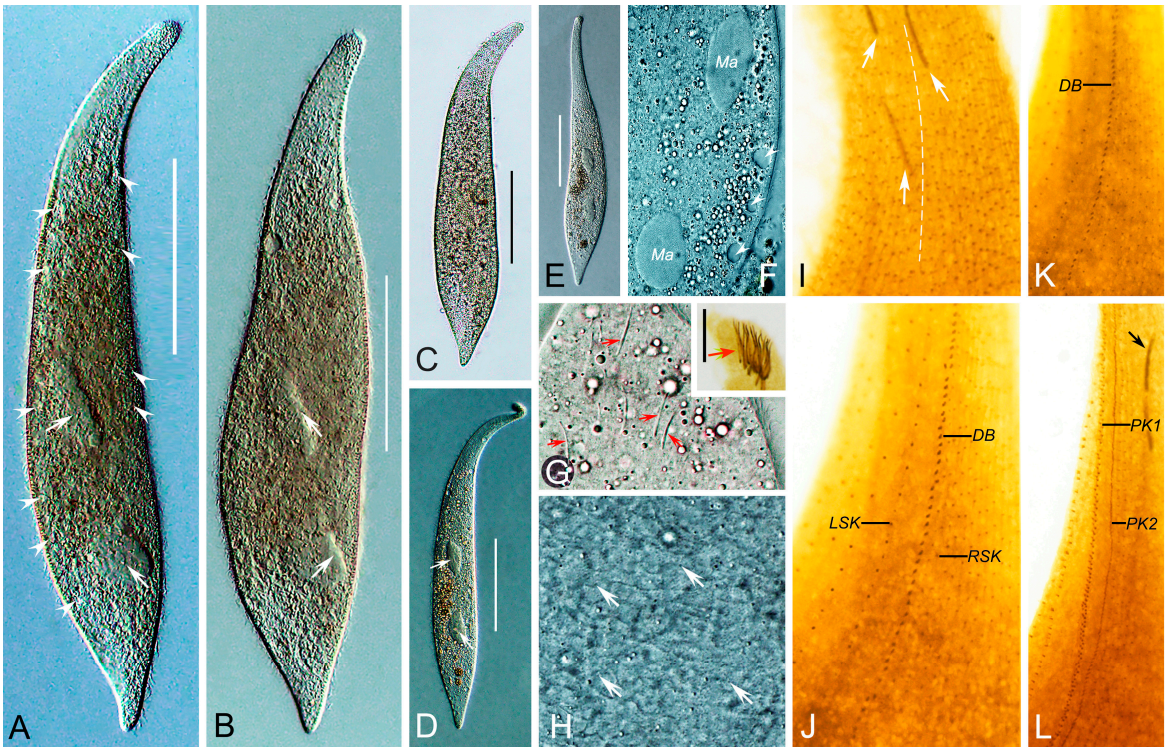

Figure 4. *Amphileptus sinicus* sp. nov. micrographs from a living cell (**A**–**H**) and after protargol staining (**I**–**L**). (**A**,**B**) Left views of two typical individuals, arrowheads indicate the contractile vacuoles on the ventral and dorsal margins, arrows indicate the ellipsoidal macronuclear nodules; (**C**–**E**) shape variants, arrows indicate the ellipsoidal macronuclear nodules; (**F**) details of the nuclear apparatus and the contractile vacuoles (arrowheads); (**G**) detail of the scattered narrowly cuneate-shaped extrusomes (arrows); (**H**) detail of the dot-like cortical granules (arrows); (**I**) detail of the anterior of right side, white dashed line marks the anterior suture, arrows mark the extrusomes; (**J**) detail of the right and left ciliary pattern; (**K**) detail of the dorsal brush kinety; (**L**) detail of the oral ciliary pattern. Abbreviations: DB, dorsal brush; LSK, left somatic kinety; Ma, macronuclear nodules; PK1, perioral kinety 1; PK2, perioral kinety 2; RSK, right somatic kinety. Scale bars: 120 μm.

Table 1. Morphometric characteristics of *Amphileptus sinicus* sp. nov. (first line), *Amphileptus piscinarius* sp. nov. (second line) based on protargol-stained specimens [a].

Character	Min	Max	Mean	Median	SD	CV	*n*
Cell length (μm)	255	612	413.5	409	89.25	21.6	24
	121	251	182.9	183	35.8	19.6	29
Cell width (μm)	83	175	129.1	128	26.49	20.5	24
	21	60	34.3	33	8.25	24.0	29
Number of right somatic kineties [b]	42	61	49.9	50	4.97	10.0	23
	24	28	25.1	25	1.06	4.2	29
Number of left somatic kineties [c]	8	10	8.9	9	0.74	8.3	24
	6	8	7.0	7	0.80	11.4	23
Number of dorsal brush dikinetids	67	156	112.2	116	23.32	20.8	18
	23	68	38.0	35	12.07	31.8	11
Number of macronuclear nodules	2	2	2.0	2	0	0	23
	1	2	1.9	2	0.26	13.4	29
Length of macronuclear nodule (μm)	48	106	73.0	74	16.56	22.7	23
	18	36	26.5	26	5.08	19.1	29
Width of macronuclear nodule (μm)	30	82	50.5	50	13.25	26.2	23
	14	31	26.4	26	5.15	19.5	29
Diameter of micronucleus (μm)	4	4	4.0	4	0	0	1
	-	-	-	-	-	-	-

[a] Abbreviations: CV, coefficient of variation (%). Max, maximum. Min, minimum. *n*, number of specimens investigated. SD, standard deviation. -, data not available. [b] Perioral kinety 2 included. [c] Perioral kinety 1 and dorsal brush kinety included.

ZooBank registration number: urn:lsid:zoobank.org:act:BEFD85A1-83F6-4F04-B967-9D8746F41870.

Amphileptus piscinarius sp. nov. (Figures 5 and 6; Table 1)

ZooBank registration number: urn:lsid:zoobank.org.act:1339C450-B9DD-4320-BEAD-47F6045185B8.

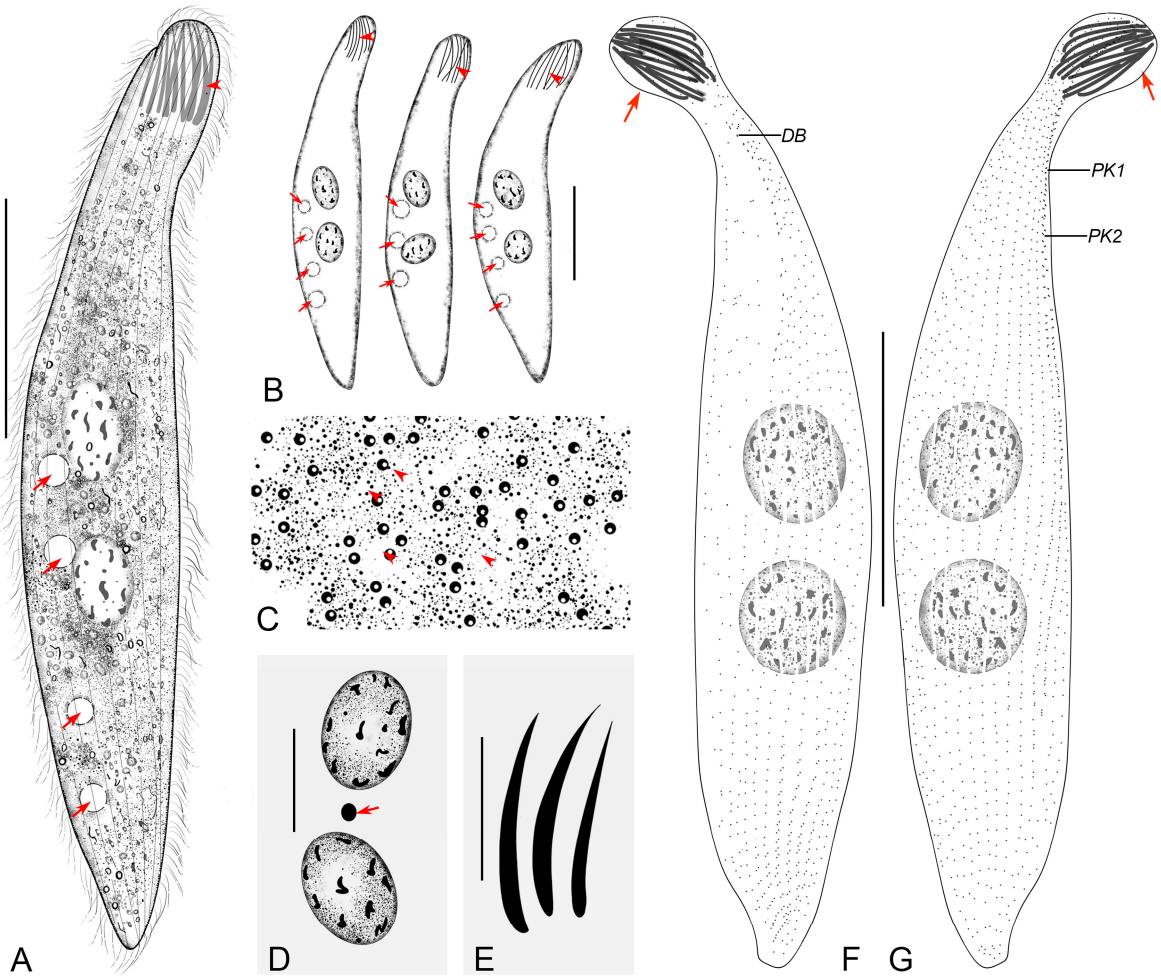

Figure 5. *Amphileptus piscinarius* sp. nov., drawings of a living cell (**A**–**E**), and after protargol staining (**F**,**G**). (**A**) Left view of a representative individual, arrows indicate the contractile vacuoles on the ventral margin, arrowhead denotes the apical extrusome group; (**B**) shape variants, arrows denote the contractile vacuoles on the ventral margins, arrowheads indicate the apical extrusome group; (**C**) dot-like cortical granules (arrowheads); (**D**) nuclear apparatus, arrow indicates the micronucleus; (**E**) narrowly ovate extrusomes; (**F**) ciliary pattern of the dorsal side and portion of right side, arrow indicates the apical extrusome group; (**G**) ciliary pattern of the right side, arrow indicates the apical extrusome group. Abbreviations: DB, dorsal brush; PK1, perioral kinety 1; PK2, perioral kinety 2. Scale bars: 40 µm in (**A**,**B**); 30 µm in (**D**); 13 µm in (**E**); 40 µm in (**F**,**G**).

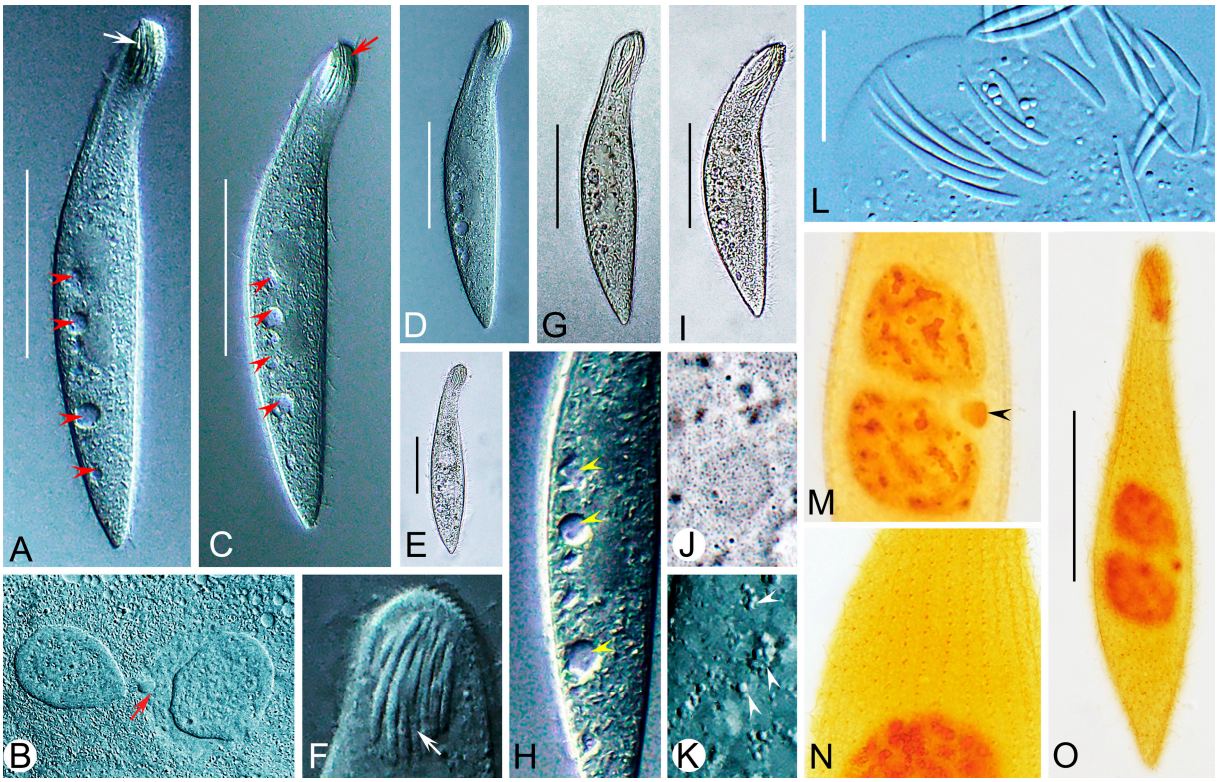

Figure 6. *Amphileptus piscinarius* sp. nov., micrographs of a living cell (**A**–**L**) and after protargol staining (**M**–**O**). (**A**,**C**) Left view of two typical individuals, arrowheads point to the contractile vacuoles on the ventral margins, arrows indicate the apical extrusomes groups; (**B**) ellipsoidal macronuclear nodules, arrow indicates the micronucleus; (**D**,**E**,**G**,**I**) shaped variants; (**F**) detail of the apical extrusome group (arrow); (**H**) detail of the contractile vacuoles on the ventral margin (arrowheads); (**J**) dot-like cortical granules; (**K**) detail of the cytoplasm, arrowheads indicate the granules; (**L**) detail of the narrowly ovate extrusomes; (**M**) nuclear apparatus, arrowhead indicates the micronucleus; (**N**) detail of the anterior of right side; (**O**) ciliary pattern of the right side. Scale bars: 60 µm in (**A**,**C**–**E**,**G**,**I**,**O**); 13 µm in (**L**).

3.1.1. *Amphileptus sinicus* sp. nov.

Diagnosis. Cell lanceolate, 330–490 × 65–90 µm in vivo; two macronuclear nodules; contractile vacuoles distributed along the ventral and dorsal margins; extrusomes very narrowly cuneate, arranged in an apical group and scattered in the cytoplasm; cortical granules dot-like and colorless; 8–10 left and 42–61 right kineties; right anterior suture; freshwater habitat.

Type material. A protargol slide with the holotype specimen circled by black ink and one further protargol slide with paratype specimens have been deposited in the Laboratory of Protozoology, Ocean University of China, with registration numbers ZGAT20210315-1, ZGAT2021031501-2, respectively.

Type locality. A fish farming pond in Lake Weishan Wetland, China (N 34°46′19.85″, E 117°09′45.93″).

Etymology. The species name *sinicus* (Latin adjective, Chinese) refers to the fact that this species was first discovered in China.

Description. Cell about 330–490 × 65–90 µm in vivo, slightly contractile, elongate–lanceolate in lateral view, cell laterally compressed, with anterior end rounded (Figures 3A,B and 4A–E). Nuclear apparatus in cell half to posterior cell half. Invariably two macronuclear nodules, individual nodules ellipsoidal, about 35–50 × 50–70 µm in vivo (Figures 3A,B and 4A–F). Micronucleus not observed in vivo but recorded after protargol staining as 4 µm in diameter. About 10–12 contractile vacuoles arranged in two rows along ventral and

dorsal margins, 6 μm in diameter, pulsating every 30 s (Figures 3A and 4A,F). Extrusomes very narrowly cuneate, sometimes slightly curved, about 9–12 μm in vivo, some attached to anterior end of cell forming an apical group, others scattered throughout cytoplasm (Figures 3A,B,D and 4G). Cortex very flexible; cortical granules dot-like, colorless, about 1 μm across, densely spaced on the right side (Figures 3C and 4H). Cytoplasm contains numerous small granules which render cell opaque (Figures 3A and 4A–F). Locomotion by gliding on substrate or occasionally by swimming while rotating about long axis.

Right somatic cilia about 9–11 μm long, very densely arranged on right side (Figures 3A and 4A,B,H), whereas left ones short and sparsely distributed, difficult to detect in vivo. Ciliary pattern as shown in Figures 3F,G and 4I–L. About 42–61 right kineties including perioral kinety 2, intermediate kineties progressively shortened anteriorly forming a suture (Figures 3G and 4I); 8–10 left kineties including perioral kinety 1 and dorsal brush (Figures 3F and 4K,J). Dorsal brush kinety composed of 67–156 densely spaced dikinetids in anterior cell third and of monokinetids in posterior two thirds (Figure 3F).

Two perioral kineties. Perioral kinety 1, on the left of oral slit, consists of densely spaced dikinetids in anterior half of cell and continues as densely spaced monokinetids. Perioral kinety 2, on the right of oral slit, consists of densely spaced dikinetids in anterior two thirds and continues as densely spaced monokinetids (Figures 3E,G and 4L). Nematodesmata not observed in vivo or after protargol staining.

3.1.2. *Amphileptus piscinarius* sp. nov.

Diagnosis. Cell elongate–lanceolate, about 140–210 × 20–30 μm in vivo; two globular to ellipsoidal macronuclear nodules; three to four contractile vacuoles distributed along the ventral margin; extrusomes very narrowly ovate, only arranged in an apical group; cortical granules dot-like and colorless (1 μm); 6–8 left and 24–28 right kineties; two perioral kineties; freshwater habitat.

Type material. A protargol slide with the holotype specimen circled by black ink and one further protargol slide with paratype specimens have been deposited in the Laboratory of Protozoology, Ocean University of China, with registration numbers ZGAT20210404-1, ZGAT2021040401-2, respectively.

Type locality. A fish farming pond in Lake Weishan Wetland, China (N 34°46′11.16″, E 117°09′59.04″).

Etymology. The species name "piscinarius" (Latin adjective for belonging to a fish pond) refers to the sampling location of this species.

Description. Cell about 140–210 × 20–30 μm in vivo; non-contractile; cell elongate–lanceolate; anterior end rounded, neck region inconspicuous; posterior end slightly pointed; no distinct tail region (Figures 5A,B and 6A,C–E,G,I). Nuclear apparatus centrally located. Invariably two macronuclear nodules; nodules globular to ellipsoidal, about 13–30 × 17–37 μm in vivo. Single micronucleus, about 5 × 3 μm in vivo (Figures 5A,D and 6B,M). Three to four contractile vacuoles along the posterior half of ventral margin, about 5–7 μm in diameter, pulsating every 20 s (Figures 5A,B and 6A,C,H). Extrusomes very narrowly ovate, sometimes slightly curved, about 11–19 μm in vivo, solely attached to anterior end of cell forming an apical group, no scattered extrusomes detected in the cytoplasm (Figures 5A,B,E–G and 6A,C,D,G,I,L). Cortex very flexible; cortical granules dot-like, colorless, about 0.5–1.0 μm in vivo (Figures 5C and 6J). Cytoplasm contains numerous small granules rendering cell opaque (Figures 5A and 6K). Swims fast while rotating about longitudinal axis, never observed gliding on substrate.

Right somatic cilia about 6–7 μm long, very densely arranged on right side (Figure 5A); whereas left somatic cilia sparsely distributed on the left side and therefore usually undetectable in vivo. Ciliary pattern as shown in Figures 5F,G and 6N,O. About 24–28 right kineties including perioral kinety 2 (Figure 5F,G); 6–8 left kineties including perioral kinety 1 and dorsal brush (Figure 5F,G). Dorsal brush kinety composed of 23–68 densely spaced dikinetids in anterior cell third and of monokinetids in posterior two thirds (Figure 5F).

Two perioral kineties, perioral kinety 1 and perioral kinety 2, located on the left and right of oral slit, respectively (Figure 5G). Perioral kinety 1 is composed of densely spaced dikinetids in upper third of cell length and of monokinetids in posterior part. Perioral kinety 2, on the right of oral slit, consists of densely spaced dikinetids in anterior half of cell and continues as densely spaced monokinetids. Nematodesmata not observed in vivo or after protargol staining.

3.2. 18S rRNA Gene Sequences

In the present study, novel 18S rRNA gene sequences were obtained from each of the two new species.

The 18S rRNA gene sequences of *Amphileptus sinicus* sp. nov. are deposited in GenBank with the following information: length 1591 bp, GC content 42.36%, accession number PP768119.

The 18S rRNA gene sequences of *Amphileptus piscinarius* sp. nov. are deposited in GenBank with the following information: length 1542 bp, GC content 42.09%, accession number PP768120.

The sequence similarities among other *Amphileptus* species range from 87.3% to 100%, as shown in Figures 2B and 7.

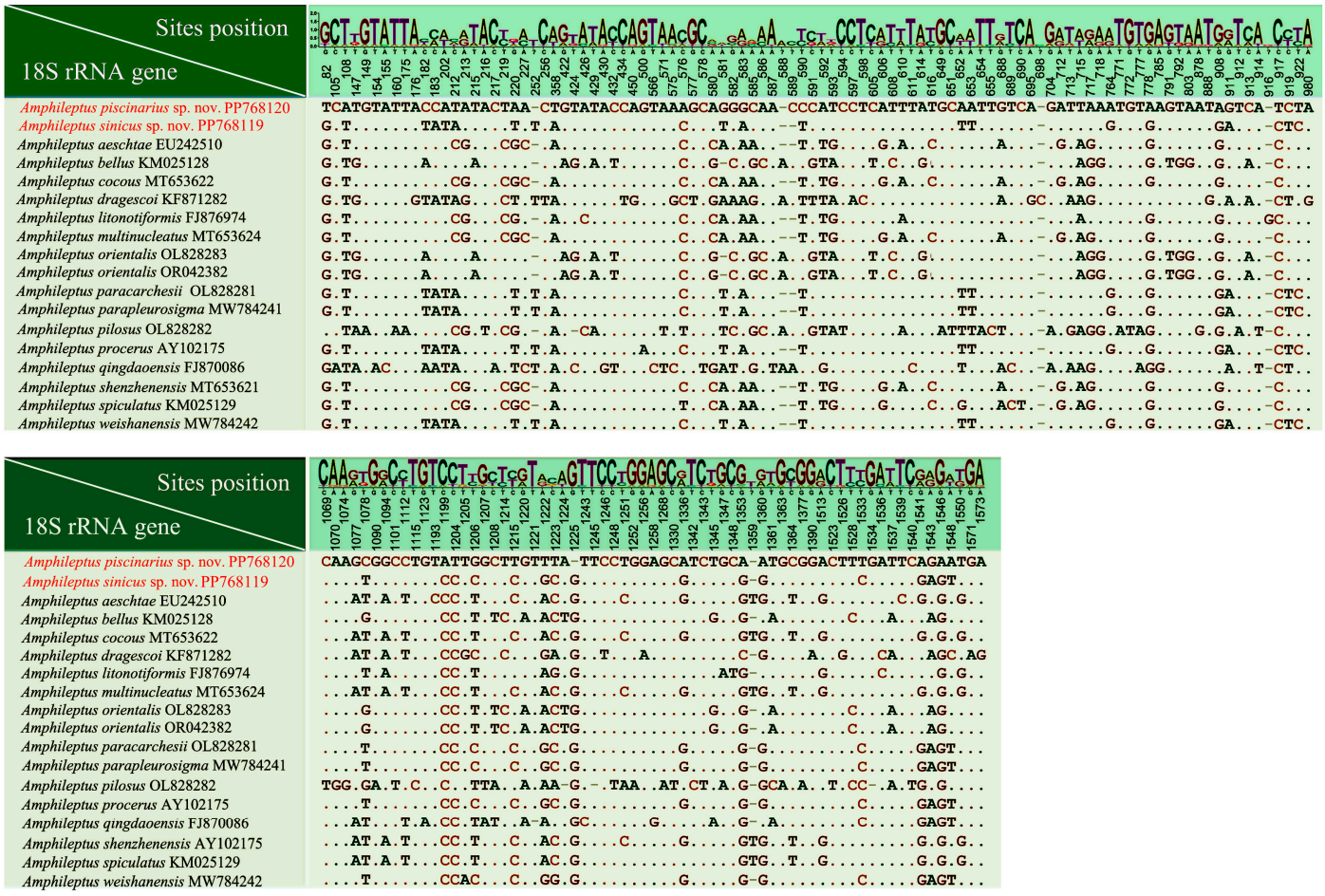

Figure 7. Nucleotide differences between *Amphileptus* spp. based on 18S rRNA gene sequences. Missing sites are indicated by dashes (-). Filled circles represent the same position of nucleotides.

3.3. Phylogenetic Positions of Two Species

The ML and BI trees based on 18S rRNA gene data have mostly consistent topologies, therefore only the ML tree is presented (Figure 2). The family Amphileptidae is separated into six clades. In Clade 1, two *Pseudoamphileptus* species form a fully supported subclade and then clustered with *Amphileptus qingdaoensis* (FJ870086) with the strongest support. Clade 2 comprises five *Amphileptus* including *Amphileptus sinicus* sp. nov., with strong statistical support (98% ML, 1.00 BI), which form a sister group with Clade 1 with low variable support (39% ML, 0.68 BI). Clade 3 only consists of *Amphileptus dragescoi*, whose position is ambiguous (41% ML, *). *Amphileptus bellus* and two populations of *A. orientalis* from Clade 4 show the strongest support, and then together group with *A. pilosus* with variable support (63% ML, 0.99 BI). Clade 5 comprises six *Amphileptus* species, which is sister to Clade 4 with low support (24% ML, *). *Amphileptus piscinarius* sp. nov. forms Clade 6, located outside of the assemblage of Clade 4 and Clade 5 with low support (50% ML, 0.81 BI).

4. Discussion

4.1. Comparison of Amphileptus sinicus sp. nov. with Similar Species

Amphileptus Ehrenberg, 1830, the oldest genus within Pleurostomatida, was characterized by a distinct right anterior suture [20]. Besides the right anterior suture, other amphileptids (except *Amphileptus*) possess more distinctive features, such as: *Apoamphileptus* has a postoral suture; *Amphileptiscus* has a "spoon"-shaped apex at the anterior end. Previous studies suggest that the number and distribution of the contractile vacuoles are useful live features for species identification within the genus *Amphileptus* (Ehrenberg, 1830) with variations including: (1) single contractile vacuole in different locations: subterminally located; terminally located; located in ventral margin or in dorsal margin, etc.; (2) multiple contractile vacuoles: located on the dorsal margin; located on the ventral margin; located on the both dorsal and ventral margins; scattered, etc. Concerning multiple contractile vacuoles arranged along both the dorsal margin and the ventral margin, nine species should be compared with *Amphileptus sinicus* sp. nov.: *A. parapleurosigma* Zhang et al., 2022; *A. pleurosigma* (Stokes, 1884) Foissner, 1984; *A. polymicronuclei* Li, 1990; *A. proceroformis* Song & Wilbert, 1989; *A. procerus* (Penard, 1922) Song & Wilbert, 1989; *A. quadrinucleatus* (Dragesco & Njiné, 1971) Dragesco & Dragesco-Kernéis, 1986; *A. salignus* Chen et al., 2011; *A. fusiformis* Song & Wilbert, 1989; *A. weishanensis* Zhang et al., 2022 (Table 2).

Amphileptus sinicus sp. nov. can be distinguished from *A. fusiformis*, *A. polymicronuclei* and *A. salignus* by having a distinct apical extrusome group (vs. absent in others). Moreover, the new species has more somatic kineties (8–10 left and 42–61 right kineties in *Amphileptus sinicus* sp. nov. vs. 6 left and 10–14 right kineties in *A. fusiformis*; 23–25 right kineties in *A. polymicronuclei*; 4 left and 24–29 right kineties in *A. salignus*) and a larger cell size (330–490 μm in *Amphileptus sinicus* sp. nov. vs. 45–60 μm in *A. fusiformis*; 230 μm in *A. polymicronuclei*; 180–360 μm in *A. salignus*) [58–60].

Amphileptus quadrinucleatus and *A. weishanensis* have a large cell size as well. However, *Amphileptus sinicus* sp. nov. can be distinguished from them by having more left kineties (8–10 vs. 5), and the narrowly cuneate extrusomes, (vs. filiform extrusomes in *A. quadrinucleatus* and *A. weishanensis*). Moreover, *A. sinicus* sp. nov. has two macronuclear nodules whereas *A. quadrinucleatus* and *A. weishanensis* have multiple macronuclear nodules [38,61].

Amphileptus sinicus sp. nov. closely resembles *A. parapleurosigma*, *A. pleurosigma*, *A. proceroformis* and *A. procerus* by having an apical extrusome group, scattered extrusomes and living in freshwater habitats. However, *Amphileptus sinicus* sp. nov. can be distinguished from them by having more right kineties (42–61 right kineties in *Amphileptus sinicus* sp. nov. vs. 19–24 right kineties in *A. parapleurosigma*; 25–35 right kineties in *A. pleurosigma*; 14–26 right kineties in *A. proceroformis*; 25–40 in *A. procerus*) [15,38,58].

Table 2. Comparison of *Amphileptus sinicus* sp. nov. with similar congeners (two rows of CVs) [a].

Species	Cell Length In Vivo	No. of LK/RK	No. of Ma	Shape of Ex	Distribution of Ex	Apical Group	Habitat	Reference
Amphileptus sinicus sp. nov.	330–490	8–10/42–61	2	Narrowly cuneate	Anterior end; scattered	Present	FW	Present work
A. parapleurosigma	180–370	4–6/19–24	2	Clavate	Anterior end; scattered	Present	FW	[38]
A. pleurosigma	150–450	4–6/25–35	2	Thorn-shaped	Anterior end; scattered	Present	FW	[15]
A. polymicronuclei	230	-/23–25	2	-	Oral slit	Absent	FW	[59]
A. proceroformis	120–350	5–6/14–26	2	Rod-shaped	Anterior end; scattered	Present	FW	[58]
A. procerus	200–800	7–13/25–40	2	Rod-shaped	Anterior end; scattered	Present	FW	[15]
A. quadrinucleatus	200–370	5/30–34	4	Filiform [b]	Oral slit; scattered	Absent	SW	[61]
A. salignus	180–360	4/24–29	2	Bar-shaped; short-bar-like	Oral slit; scattered	Absent	BW	[60]
A. fusiformis	45–60	6/10–14	2	Rod-shaped	Scattered	Absent	FW	[58]
A. weishanensis	560–780	5/56–61	3–9	Filiform	Anterior end; oral slit; scattered	Present	FW	[38]

[a] Abbreviations: CV, contractile vacuole; Ex, extrusomes; FW, freshwater; LK, left kineties; RK, right kineties; SW, seawater. [b] Data from illustrations.

4.2. Comments on *Amphileptus piscinarius sp. nov.*

Amphileptus piscinarius sp. nov., a planktonic species, is characterized by having a distinct large apical extrusome group and ventrally positioned contractile vacuoles. In terms of these two characteristics, five species can be compared with it, namely *A. carchesii* Stein, 1867, *A. ensiformis* Song & Wilbert, 1989, *A. gui* Lin et al., 2005, *A. inquieta* (Biernacka, 1963) Carey, 1992; *A. paracarchesii* Zhang et al., 2022 (Table 3).

Table 3. Comparison of *Amphileptus piscinarius* sp. nov. with similar congeners [a].

Species	Cell Length In Vivo	No. of LK/RK	No. of Ma	No. of CV	Shape of Ex	Distribution of Ex	Habitat	Reference
A. piscinarius sp. nov.	140–210	6–8/24–28	2	3–4	Narrowly ovate	Anterior end	FW	Present work
A. carchesii	200–360	5/45	4	10	Thorn-shaped	Anterior end; scattered	FW	[15]
A. ensiformis	100–120	5–6/18–22	2	4 [b]	Rod-shaped [b]	Anterior end	FW	[58]
A. gui	150–300	7–11/37–50	2	3–7	Bar-shaped	Anterior end; scattered	SW	[40]
A. inquieta	170–200	-	2	4	-	-	SW	[62]
A. paracarchesii	185–380	4–6/44–50	4	10	Narrowly ovate	Anterior end; scattered	FW	[39]

[a] Abbreviations: CV, contractile vacuole; Ex, extrusomes; FW, freshwater; LK, left kineties; RK, right kineties; SW, seawater. [b] Data from illustrations.

Amphileptus piscinarius sp. nov. can be separated from *A. carchesii* and *A. paracarchesii* by consistently having two macronuclear nodules (vs. four in *A. carchesii* and *A. paracarchesii*). Furthermore, *Amphileptus piscinarius* sp. nov. has fewer right kineties (24–28 vs. 45 in *A. carchesii*, 44–50 in *A. paracarchesii*) [15,39].

Amphileptus piscinarius sp. nov. can be separated from *A. ensiformis* by having more left and right kineties (6–8 left and 24–28 right kineties in *Amphileptus piscinarius* sp. nov. vs. 5–6 left and 18–22 right kineties in *A. ensiformis*) [58].

Amphileptus piscinarius sp. nov. can be distinguished from *A. gui* by having fewer kineties (6–8 left and 24–28 right kineties in *Amphileptus piscinarius* sp. nov. vs. 7–11 left and 37–50 right kineties in *A. gui*). Furthermore, *Amphileptus piscinarius* sp. nov. does not have scattered extrusomes in its cytoplasm but *A. gui* does [40].

Amphileptus inquieta is very similar to *A. piscinarius* sp. nov. in cell size and number of contractile vacuoles. Though the infraciliature details of *A. inquieta* are as yet unknown, it

can be distinguished from *A. piscinarius* sp. nov. by its rounded posterior end (vs. pointed end in *A. piscinarius* sp. nov.) and seawater habitat (vs. freshwater habitat) [62].

4.3. Phylogenetic Analyses

Based on the phylogenetic analyses presented here, the family Amphileptidae is consistently monophyletic, but the genus *Amphileptus* is non-monophyletic with *A. qingdaoensis* clustering with two *Pseudoamphileptus* species, which is consistent with previous studies [26,28,34,37–39,46,63–65].

In the 18S rRNA gene tree presented here, *Amphileptus sinicus* sp. nov., *A. parapleurosigma*, *A. procerus* and *A. weishanensis* cluster together with strong statistical support (98% ML, 1.00 BI), which is supported by their morphometric features: the distribution of extrusomes forming an apical group; multiple contractile vacuoles. It is noteworthy that the partial 18S rRNA gene sequences of *Amphileptus sinicus* sp. nov., *A. parapleurosigma* and *A. paracarchesii* share a 100% similarity match of their reported 18S rRNA gene. *Amphileptus sinicus* sp. nov. and *A. parapleurosigma* were both collected from Lake Weishan Wetland, and they share several morphological features, e.g., possessing two rows of contractile vacuoles and having apical extrusome groups. But they can be distinguished from each other morphologically by the number of kineties: 8–10 left and 42–61 right kineties in *Amphileptus sinicus* sp. nov. vs. 4–6 left and 19–24 right kineties in *A. parapleurosigma*. A small variation is also found in their cell size (330–490 μm in *Amphileptus sinicus* sp. nov. vs. 180–370 μm in *A. parapleurosigma*). It is worth noting here the possibility of cryptic species in this example (as well as ciliates in general), especially with regard to two apparently different cells sharing an exact molecular signature of one gene marker. To what extent there is morphological plasticity between similar appearing species within *Amphileptus* awaits further investigations. As more sequences become available in databases, and as alternate gene markers are utilized for comparisons, the exact divergences between these species and ones awaiting discovery can be compared. The importance of detailed morphological descriptions such as those presented here remain vital for the use of future researchers examining advancing molecular markers. Equally important is combining these descriptions with sequencing of the exact species/cell being described. Misidentifications uploaded to public databases such as GenBank can hinder future researchers for generations, such that where clonal strains are not possible or practical, freshly sampled environmental material must be confidently identified prior to DNA analysis.

Amphileptus sinicus sp. nov. and *A. paracarchesii* share a similar cell shape. However, *A. sinicus* sp. nov. can be distinguished from *A. paracarchesii* by having more left kineties (8–10 vs. 4–6 in *A. paracarchesii*), fewer macronuclear nodules (two vs. four in *A. paracarchesii*) and two rows of contractile vacuoles (vs. one row of the contractile vacuoles along the dorsal margin in *A. paracarchesii*). Additionally, *A. paracarchesii* has a lateral fossa (groove) in the posterior cell portion, which is absent in *Amphileptus sinicus* sp. nov. [39]. Previous studies of *Amphileptus* though have found species with differences in only a single 18S rRNA gene base pair or no differences in these bases but still found apparently clear differences of morphology [38,39], with these instances also occurring in hypotrich ciliates [66]. The rates of morphological and molecular evolution between various higher species have been discussed in many studies [67–70]. A previous study found that the rates of molecular and morphological change are considered to be effectively disassociated [68]. Additional previous studies have found that the molecular evolution of different species can vary substantially and can be considered as an essentially stochastic process [68,69]. Irrespective of this, it is well established that the 18S rRNA gene is highly conserved [71], and therefore may not always be an appropriate marker to display a species-specific signal for some ciliates [39,66]. Consequently, more gene markers such as the ITS1-5.8S-ITS2, 28S rRNA gene and CO1 gene can be utilized in future research that would help to confirm or reject a novel species.

Amphileptus piscinarius sp. nov. is positioned on a separated branch, which branches basally in the assemblage of two clades (Clade 4 and Clade 5) with poor statistical supports

(50% ML, 0.81 BI). However, *Amphileptus piscinarius* sp. nov. clearly differs from all species (within Clades 4 and 5) by the distribution of extrusomes (having a well-developed apical extrusome group and no scattered extrusomes in the cytoplasm). Furthermore, *Amphileptus piscinarius* sp. nov. is a typical freshwater planktonic species, never gliding on substrate. Due to the low statistical support for the assemblage, the phylogenetic positions of *Amphileptus piscinarius* sp. nov. and relationship between *Amphileptus piscinarius* sp. nov. and other species remaining ambiguous, further molecular data from future studies will help to resolve the phylogenetic position of this species.

4.4. The Biogeography of the Genus Amphileptus

Amphileptus species are commonly found in a variety of aquatic environments including marine, freshwater and brackish water habitats. Previous studies have shown that *Amphileptus* spp. are widely distributed in Europe, America, Africa and China. However, in the past two decades, studies on the taxonomy and systematics of pleurostomatids were highly biased in China as most studies were based only on marine and brackish habitats. The evolutionary and phylogenetic relationships within this group would benefit from further explorations including additional molecular and detailed morphological information. Thus, studies such as this on the freshwater pleurostomatids in China and beyond are necessary, including morphological information, ecological data and molecular information from diverse habitats.

5. Conclusions

Increased sampling continues to increase the diversity of species (approximately 50) in the genus *Amphileptus*, which are commonly found from a variety of habitats. Due to the similarity of morphological features of species in this genus, it is difficult to separate them based on living characteristics alone. Since other genera in the family Amphileptidae are yet to have their molecular sequences described, the ultimate taxonomical positioning remains to be formulated. Here, we described two *Amphileptus* species from Lake Weishan Wetland, northern China using modern taxonomic and molecular techniques to provide underpinning for future work investigating known and new species yet to be discovered, and ultimately allowing for a better understanding of the placement of this and other genera in the family. Since some *Amphileptus* species cannot be easily separated by their 18S rRNA gene sequence alone, investigating additional gene markers could provide better clarity into the phylogenetic positioning of species within the Amphileptidae.

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