



# Species Diversity of the Pleurostomatid Ciliate Genus *Amphileptus* (Ciliophora, Haptoria), With Notes on the Taxonomy and Molecular Phylogeny of Three Species

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*Amphileptus* is one of the largest genera of pleurostomatid ciliates and its species diversity has been reported in various habitats all over the world. In the present work, we review its biodiversity based on data with reliable morphological records. Our work confirms that there are 50 valid *Amphileptus* species, some of which have a wide range of salinity adaptability and diverse lifestyles. This genus has a high diversity in China but this might be because of the relatively intensive sampling. Phylogenetic analyses based on SSU rDNA sequence data verify the non-monophyly of the genus *Amphileptus*. Furthermore, two new and one poorly known *Amphileptus* species, namely *A. shenzhenensis* sp. n., *A. cocous* sp. n., and *A. multinucleatus* Wang, 1934, from coastal habitats of southern China were investigated using morphological and molecular phylogenetic methods. These three species are highly similar based on their contractile vacuoles and macronuclear nodules. However, they can be discriminated by details of their living morphology and somatic kineties. We also propose two new combinations, *Amphileptus polymicronuclei* (Li, 1990) comb. n. (original combination *Hemiophrys polymicronuclei* Li, 1990) and *Amphileptus salimicus* (Burkovsky, 1970b) comb. n. (original combination *Hemiophrys salimica* Burkovsky, 1970b).

**Keywords:** biodiversity, *Hemiophrys*, Litostomatea, new species, phylogeny, SSU rDNA

## INTRODUCTION

Ciliated protozoa (ciliates) are a highly differentiated and diverse group of eukaryotic unicellular organisms which are common in a wide range of habitats where is sufficient water for their survival (Carey, 1992; Foissner, 1999; Wilbert and Song, 2005; Lynn, 2008; Foissner and Hawksworth, 2009; Song et al., 2009; Vďačný and Foissner, 2012; Gao et al., 2016; Liu et al., 2017, 2019, Liu M.J. et al., 2020; Liu W.W. et al., 2020; Qu et al., 2018; Hu et al., 2019; Ma et al., 2019; Fan and Pan, 2020).

Pleurostomatida Schewiakoff, 1896 are a large order within the class Litostomatea Small and Lynn, 1981 and recent studies have revealed that its species diversity is much higher than previously anticipated (Lin et al., 2009; Vd'achný et al., 2011, 2014; Vd'achný, 2015; Wu et al., 2017; Hu et al., 2019). In the last two decades, investigations in China have demonstrated that pleurostomatids have a high diversity in both marine and brackish habitats (Lin et al., 2005a,b, 2007a,b, 2008, 2009; Pan et al., 2010, 2013, 2014, 2015, 2020; Wu et al., 2013, 2014, 2015a,b, 2017). As a result of these findings, knowledge and understanding of the systematics of the pleurostomatids has greatly improved (Wu et al., 2015a, 2017).

*Amphileptus* Ehrenberg, 1830 is the oldest genus within the order Pleurostomatida and comprises over 60 nominal species reported from marine (Wang, 1934; Dragesco, 1965; Song, 1991; Carey, 1992; Lin et al., 2005a,b, 2007a), brackish waters (Pan et al., 2014; Wu et al., 2014, 2015b), and freshwater habitats (Wang and Nie, 1933; Wang, 1940; Curds, 1982; Song and Wilbert, 1989; Li, 1990) all over the world. Most are free-living but some live as parasites on the skin and gills of certain freshwater fishes and tadpoles (Wenrich, 1924; Chen, 1955; Mitchell and Smith, 1988; Masoumian et al., 2005). *Amphileptus* is generally defined by the following combination of characters: (1) a single anterior suture formed by the right somatic kineties; (2) the presence of two rows of perioral kineties [three rows were detected in a single species, *A. yuianus*, by Lin et al. (2005b); molecular data are needed to confirm its generic classification]; (3) extrusomes not distributed along the dorsal margin, and (4) the absence of a spoon-shaped apex in the anterior end of the body (Foissner, 1977, 1984; Song and Wilbert, 1989; Foissner and Leipe, 1995; Lin et al., 2007a). Species of *Amphileptus* have a high degree of morphological similarity *in vivo*, and many have not been studied using modern methods such as silver staining. This has resulted in numerous examples of misidentifications and/or synonyms and homonyms within the *Amphileptus-Litonotus-Loxophyllum* complex, especially among the many nominal species described before the 1960s (Kahl, 1931, 1933; Wang and Nie, 1932; Wang, 1934, 1940; Dragesco, 1960; Vuxanovici, 1960, 1961). Since the ciliary pattern as revealed by silver staining is of great importance for species identification, there is an urgent need to redescribe those that are currently known only from *in vivo* observation.

*Amphileptus* has long been considered to be monophyletic based on morphological information (Fryd-Versavel et al., 1975; Foissner, 1977, 1984; Corliss, 1979; Song and Wilbert, 1989). However, recent studies based on the molecular data have indicated that the molecular and morphological data are not concordant and the molecular data suggest that the genus *Amphileptus* is non-monophyletic (Pan et al., 2014; Wu et al., 2015b). In addition, most congeners within this genus are very similar in terms of their body shape, the number and position of contractile vacuoles, and other aspects of their living morphology. Therefore, more detailed morphological information and molecular data obtained from expanded taxon sampling are necessary.

In this paper we: (1) briefly review previous studies of the species diversity of the genus *Amphileptus*; (2) provide a checklist of valid species including synonyms following analyses

of nomenclatural problems, and (3) reconstruct the molecular phylogeny of the family Amphileptidae Ehrenberg, 1830 and the genus *Amphileptus* based on all reliable small subunit (SSU) rDNA sequences from the NCBI/GenBank database. In addition, we investigate three morphologically similar *Amphileptus* species from coastal waters of southern China. After detailed comparisons, they were identified as *Amphileptus multinucleatus* Wang, 1934, *Amphileptus shenzhenensis* sp. n. and *Amphileptus cocous* sp. n.

## MATERIALS AND METHODS

### Sample Collection, Observation, and Identification

All samples were collected from coastal waters at two sites in southern China using 250 ml wide-mouth bottles after gently stirring the water. *Amphileptus multinucleatus* Wang, 1934 and *A. cocous* sp. n. were collected on 19 December 2011 and 27 October 2011, respectively, from Daya Bay mangrove wetland in Huizhou (22°41' N, 114°23' E). *Amphileptus shenzhenensis* sp. n. was isolated on 13 April 2011 from Futian mangrove wetland in Shenzhen (22°38' N, 114°06' E). Each species was cultivated at room temperature (~25°C) in habitat water in Petri dishes with rice grains to enrich the growth of bacteria as a food source for the ciliates.

Observations of living cells were executed with bright field and differential interference contrast microscopy. The number, size and location of contractile vacuoles were recorded based on live observations. The protargol staining method according to Wilbert (1975) was used to reveal the ciliary pattern. Living cells were examined at 100–1,000 × magnifications. Measurements of stained specimens were performed at a magnification of 1,000×. Drawings of stained specimens were conducted with the help of a camera lucida at a magnification of 1,000×. Classification and terminology are according to Vd'achný et al. (2015) and Wu et al. (2017).

### DNA Extraction, Gene Amplification, and Gene Sequencing

For each species, one or several cells taken from cultures were isolated, repeatedly washed in filtered habitat water and transferred into 45 µl ATL buffer for DNA extraction. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Shanghai, China) according to the manufacturer's protocol. SSU rDNA amplification and gene sequencing were conducted as described in Wu et al. (2013).

### Phylogenetic Analyses

In total, 36 SSU rDNA sequences of the order Pleurostomatida, representing four families and including all available and reliable sequences of the family Amphileptidae, were used to conduct the phylogenetic analyses. Apart from the three new SSU rDNA sequences provided in the present study, all other sequences used in the phylogenetic analyses were obtained from the NCBI/GenBank database (see **Figure 4** for GenBank accession

numbers). Sequences were first aligned with CLUSTAL W and further modified manually using Bioedit v.7.0. The final alignment of 1625 characters and 40 taxa, including four haptorians as outgroup taxa, were used to construct phylogenetic trees using three different methods. Maximum likelihood (ML) analysis was carried out using RaxM-HPC2 v7.2.8 (Stamatakis et al., 2008) on CIPRES Science Gateway<sup>1</sup>. The reliability of internal branches came from a majority rule consensus tree by using a non-parametric bootstrap method with 1,000 replicates. Bayesian inference (BI) analysis was conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) by using the Markov chain Monte Carlo algorithm under the GTR + G + I evolutionary model indicated by MrModeltest v.2 (Nylander, 2004), which was run for 1,500,000 generations with a sample frequency of 100 generations. The first 3,750 generations were discarded as burn-in. Maximum parsimony (MP) analysis was performed with PAUP 4.0b10 (Swofford, 2002) using the tree-bisection-reconnection algorithm and bootstrapping with 1000 replicates.

### Statistical Tree Topology Test

The Kishino-Hasegawa (KH) test (Kishino and Hasegawa, 1989) was used to test the hypothesis that the genus *Amphileptus* is monophyletic. The ML tree was generated with a constraint block, enforcing the constraint of focal group monophyly in PAUP 4.0b10 under the GTR + I + G model. The site-wise likelihoods were calculated using PAUP 4.0b10 (Swofford, 2002) for the resulting constrained and non-constrained ML topologies. The scores were then subjected to the KH test as implemented in Consel (Shimodaira and Hasegawa, 2001).

### Morphological Diversity Data Collection

The species diversity of the genus *Amphileptus* was studied based on data from the present study and published sources, mainly monographs (Kahl, 1931; Song and Wilbert, 1989; Carey, 1992; Song et al., 2009; Vd'áčný and Foissner, 2012; Hu et al., 2019) and papers on the taxonomy and biodiversity of *Amphileptus* (see Tables 1, 2 for a complete list).

## RESULTS

### Geographic Distribution of the Genus *Amphileptus*

*Amphileptus* has been found in a wide variety of habitats worldwide. To date, 50 valid species of this genus have been reported from marine (Carey, 1992; Lin et al., 2005a,b, 2007a,b), brackish (Pan et al., 2010, 2014; Chen et al., 2011), freshwater (Wang and Nie, 1933; Wang, 1940; Song and Wilbert, 1989), and terrestrial (Foissner, 1984) habitats worldwide. In freshwater habitats, species of *Amphileptus* are most commonly reported from lakes (Song and Wilbert, 1989; Li, 1990), rivers (Stokes, 1884), wastewater treatment plants (Foissner, 1984), and as parasites on the body surface and gills of certain freshwater fishes and tadpoles in North America, Asia and Europe (Wenrich, 1924; Chen, 1955; Mitchell and Smith, 1988; Masoumian et al., 2005). In

marine and brackish water habitats, species are most commonly reported from mangrove wetlands (Pan et al., 2010, 2013, 2014; Chen et al., 2011; Wu et al., 2013, 2014, 2015a,b, 2017; this study), mariculture ponds (Song, 1991; Lin et al., 2005a,b, 2007a; Song et al., 2009; Pan et al., 2014), the intertidal zones of beaches (Pan et al., 2014), and coastal marine waters (Kahl, 1931; Wang, 1934; Borrer, 1963; Dragesco, 1965; Al-Rasheid, 1996). The vast majority of *Amphileptus* species are free-living although a few are reported as parasites on the skin and gills of fish (Chen, 1955; Masoumian et al., 2005), or tadpoles (Wenrich, 1924). Of the known *Amphileptus* species more than one-third have been found in the coastal waters of China (Song et al., 2009; Hu et al., 2019). These include eight species from mariculture ponds in the coastal waters of the Bohai and Yellow seas of northern China and 11 species (12 populations) from coastal waters of the South China Sea, seven of which were isolated from mangrove wetlands. We have listed the references to reliable morphological descriptions of *Amphileptus* in Table 1. Species no longer assigned to the genus *Amphileptus*, and species of *Amphileptus* originally assigned to other genera, are listed in Table 2 along with their current names and taxonomic status.

### Morphology and Taxonomy of Three *Amphileptus* Species

Order Pleurostomatida Schewiakoff, 1896

Family Amphileptidae Bütschli, 1889

Genus *Amphileptus* Ehrenberg, 1830

#### *Amphileptus multinucleatus* Wang, 1934 (Tables 3, 4 and Figure 1)

##### *Improved Diagnosis*

Medium to large *Amphileptus*, 150–450  $\mu\text{m}$   $\times$  40–80  $\mu\text{m}$  *in vivo*; posterior end constantly twisted from left to right in mid-body region; many (40–300) macronuclear nodules; 8–12 left and 29–38 right kineties; based on live observation, several (5–10) contractile vacuoles are located ventrally in posterior 2/3 of cell; extrusomes thick bar-shaped, densely arranged along oral slit; dot-like cortical granules; brackish or marine habitat.

##### *Ecological Features (Daya Bay Population)*

Water temperature 19°C, salinity 24.5‰, pH 6.7.

##### *Voucher Material*

One voucher slide with protargol-stained specimens is deposited in the Laboratory of Protozoology, OUC, China, with registration number WL2011121901.

##### *SSU rDNA Sequence*

The SSU rDNA sequence of *Amphileptus multinucleatus* is deposited in the GenBank database with the accession number, length, and GC content as follows: MT653624, 1560 bp, 43.40%.

##### *Morphological Description Based on Daya Bay Population*

Body size highly variable *in vivo*, about 200–450  $\mu\text{m}$  long; body shape fairly stable, generally elongate-pyriform with bluntly pointed; in all individuals ( $n > 20$ ) observed *in vivo*, posterior portion perpetually twisted from left side to right side beginning at mid-dorsal region; conspicuous “neck” region (about 25%

<sup>1</sup><http://www.phylo.org>

**TABLE 1** | Species list and distribution of *Amphileptus* spp. with reliable morphological description.

Species	LB ( $\mu\text{m}$ ) <sup>a</sup>	RK/LK <sup>b</sup>	n-CV <sup>c</sup>	P-CV <sup>d</sup>	Ma <sup>f</sup>	Habitat type	Sample location	Data source
<i>A. aeschtae</i>	150–350	29–34/7–10	5–13	V <sup>e</sup>	200–300	Mariculture	Qingdao, China	Lin et al., 2007a
<i>A. affinis</i>	80–130	13–18/5 or 6	1	V	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
<i>A. agilis</i>	35–65	ca. 10/–	1	V	2	Coastal beach	United Kingdom	Carey, 1992
	30–60	–/–	1	V	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
<i>A. asetosus</i>	80–160	–/–	6–12	D <sup>f</sup>	2–6	Gulf water	White Sea	Burkovsky, 1970a
<i>A. bellus</i>	250–400	31–35/6 or 7	2–4	V	2–4	Mangrove	Huizhou, China	Wu et al., 2015b
<i>A. bivacuolatus</i>	100–130	ca. 8/–	2	V	2	Freshwater	Germany	Kahl, 1931
<i>A. branchiarum</i> *	54–70	–/–	–	V, D	2	Freshwater	Philadelphia, United States	Wenrich, 1924
	65–120	–/–	Many	–	–	Freshwater	West Azerbaijan, Iran	Masoumian et al., 2005
<i>A. carchesii</i>	150–160	–/–	–	–	–	Freshwater	Breisach, Germany	Henderson, 1905
	200–360	45/–	–	V	4	Freshwater	–	Canella, 1960
	200–360	45/–	ca. 10	V	4	Freshwater	Europe	Foissner et al., 1995
<i>A. cocous</i>	180–350	27–34/7–10	ca. 10	V	> 200	Mangrove	Huizhou, China	This study
<i>A. disciformis</i> *	32–46	–	6–8	–	2	Freshwater	China	Chen, 1956
<i>A. dragescoi</i>	90–140	12–15/5	–	T <sup>g</sup>	2	Coastal water	Zhanjiang, China	Pan et al., 2014
<i>A. eigneri</i>	100–200	14–18/6–9	1	V	2	Mariculture	Qingdao, China	Lin et al., 2007a
	190–230	16–19/9 or 10	5–15	V	2	Brackish lake	Gangwon-do, Korea	Kim and Jung, 2017
<i>A. ensiformis</i>	100–200	18–22/5 or 6	Several	V	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
<i>A. falcatus</i>	25–75	12–17/5 or 6	4–6	S	1	Freshwater	Bonn, Germany	Song and Wilbert, 1989
	–	–	1	–	–	Freshwater	Hron River, Slovakia	Vd'ačný and Rajter, 2014
<i>A. filum</i>	300–500	–/–	–	–	2	Marine	Germany	Kahl, 1931
<i>A. fusidens</i>	40–55	10–14/4 or 5	1	V	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
	42.2–103.7	10–12/1–4?	1	T?	2	Freshwater	Sulawesi, Indonesia	Fernandez-Leborans and Von Rintelen, 2007
<i>A. fusiformis</i>	45–60	10–14/6	4–7	V, D	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
	–	–	–	–	–	Freshwater	Zubrovica, Slovakia	Vd'ačný and Rajter, 2014
<i>A. gui</i>	150–300	37–50/7–11	3–7	V	2	Mariculture	Qingdao, China	Lin et al., 2005b
<i>A. gutta</i> ***	215	–/–	1	T	2	Coastal water	Amoy, China	Wang and Nie, 1932
<i>A. houi</i>	100–300	21–27/9–10	2	V	1 or 2	Mariculture	Qingdao, China	Lin et al., 2009
<i>A. incurvatus</i>	53–95	–	–	–	–	–	Germany	Maupas, 1883
<i>A. inquietus</i>	170–200	–/–	4	V	2	Coastal beach	United Kingdom	Carey, 1992
<i>A. litonotiformis</i>	120–220	16–21/7–9	1	V	2	Mariculture	Sheyang, China	Song, 1991
<i>A. loxophylliformis</i>	80–110	–/–	–	–	2	Marine	–	Dragesco, 1960
<i>A. marinus</i>	150–300	13–21/5–8	7	V	2 or 3	Coastal water	Zhanjiang, China	Pan et al., 2014
	135	–/–	5	V	2	Coastal water	Saudi Arabia	Al-Rasheid, 1996
	300	–/–	Many	V, D?	–	Coastal beach	Alligator Harbor, USA	Borror, 1963
	135	–/8	5	V	2	Coastal beach	United Kingdom	Carey, 1992
	150–300	–/–	5	V	2	Coastal water	Sylt, Germany	Kahl, 1931
<i>A. meilianus</i>	150–220	17–23/5 or 6	1	S <sup>h</sup>	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
<i>A. meleagris</i>	200–300	–/–	ca. 6	V	2	Freshwater	Germany	Kahl, 1931
<i>A. multinucleatus</i>	200–450	29–38/ 8–12	5–10	V	80–300	Mangrove	Huizhou, China	This study
	150–250	–/–	6–10	V	40–70	Coastal water	Amoy, China	Wang, 1934
<i>A. musicola</i>	ca. 130	–/ca. 8	3 or 4	V	2	Freshwater	Germany	Kahl, 1931
<i>A. niloticus</i> *	57.5–67.8	10–22	–	–	2	Freshwater	Egypt	El-Tantawy et al., 2016
<i>A. parafusidens</i>	40–90	8–15/ 4 or 5	1	V	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
	–	–	–	–	–	Freshwater	Rusovce, Slovakia	Vd'ačný and Rajter, 2014
<i>A. pectinatus</i>	ca. 200	–/ca. 10	ca. 10	V	2	Freshwater	Germany	Kahl, 1931
<i>A. piger</i>	40–80	9–11/4	1	T	2	Freshwater	Austria	Sonntag and Foissner, 2004

(Continued)

TABLE 1 | Continued

Species	LB ( $\mu\text{m}$ ) <sup>a</sup>	RSK/LSK <sup>b</sup>	n-CV <sup>c</sup>	P-CV <sup>d</sup>	Ma <sup>e</sup>	Habitat type	Sample location	Data source
<i>A. pleurosigma</i>	200–300	25–35/4–6	Several	V, D	1–3	Freshwater	Bierhaum, Austria	Foissner, 1984
	90–380	28–37/6–8	Several	V, D	2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
	150–450	25–35/4–6	6–21	V, D	1–3	Freshwater	Africa; North America	Foissner et al., 1995
<i>A. polymicronulei</i> **	150–450	25–35/4–6	Several	V, D	2	Freshwater	Slovakia	Vd'ačný and Rajter, 2014
	156.2–338.3	23–25/–	ca. 8–16	V, D	2	Freshwater	Wuhan, China	Li, 1990
<i>A. proceriformis</i>	120–350	14–26/5 or 6	Several	V, D	1 or 2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
<i>A. procerus</i>	600–800	36–47/11–15	Several	V, D	1 or 2	Freshwater	Bonn, Germany	Song and Wilbert, 1989
	200–800	25–40/–	20	V, D	–	Freshwater	Eurasia; North America	Foissner et al., 1995
<i>A. punctatus</i>	–	–	–	–	–	Freshwater	Slovakia	Vd'ačný and Rajter, 2014
	100–150	18–25/4–6	1	S	2 or 3	Lawn	Traun, Austria	Foissner, 1984
	80–150	18–25/4–6	1	S	2 or 3	Freshwater	Eurasia	Foissner et al., 1995
<i>A. quadrinuleatus</i>	–	–	–	–	–	Freshwater	Slovakia	Vd'ačný and Rajter, 2014
	400–650	30–34/ca. 5	Many	V, D	4	Marine	Carmeroun	Dragesco and Njiné, 1971
<i>A. rotundus</i>	160–200	15 or 16/–	5 or 6	V	2	Freshwater	Germany	Kahl, 1931
	–	–	Several	V, S	2	Freshwater	Turiec River, Slovakia	Vd'ačný and Rajter, 2014
<i>A. salignus</i>	180–360	24–29/4	2–7	V, D	2	Mangrove	Shenzhen, China	Chen et al., 2011
	–	–	–	–	–	Mangrove	Hong Kong, China	Chen et al., 2011
<i>A. salmicus</i> **	70–90	ca. 20–22/–	8–10	D	2	Marine	White Sea	Burkovsky, 1970b
<i>A. shenzhenensis</i>	125–250	22–27/6–8	6–12	V	>200	Mangrove	Shenzhen, China	This study
<i>A. sikorai</i>	90–200	13–18/14–17	2 or 3	D	2	Mariculture	Qingdao, China	Lin et al., 2005a
<i>A. songi</i>	200–450	20–27/10–12	3–7	V	2	Mariculture	Wendeng, China	Pan et al., 2014
<i>A. spiculatus</i>	85–150	11–14/6–8	2 or 3	V	2	Mangrove	Shenzhen, China	Wu et al., 2015b
<i>A. voracus</i> *	25–45	–/–	–	–	2	Freshwater	Iowa, USA	Davis, 1947
<i>A. wilberti</i>	180–210	15–19/7 or 8	3	V	2	Mangrove	Zhanjiang, China	Pan et al., 2014
<i>A. yuianus</i>	100–200	18–22/4	1	T	2	Mariculture	Qingdao, China	Lin et al., 2005b

<sup>a</sup>Length of body in living cells.

<sup>b</sup>Number of right kineties/number of left kineties.

<sup>c</sup>Number of contractile vacuoles.

<sup>d</sup>Position of contractile vacuoles.

<sup>e</sup>Ventral.

<sup>f</sup>Dorsal.

<sup>g</sup>Terminal.

<sup>h</sup>Subterminal.

<sup>i</sup>Number of macronucleus nodules.

\*Parasites of certain aquatic animals. \*\*New combination, transferred from *Hemiohrys* in this study. \*\*\* *Amphileptus gutta* sensu Wang and Nie, 1932 non sensu *A. gutta* (Cohn, 1866). –, N/A.

of cell length); pointed anterior end always bent toward dorsal side; laterally compressed about 3–4:1 (Figures 1A,G,H). Macronuclear nodules numerous (ca. 80–300), ovoid to elliptical in outline, about 3–7  $\mu\text{m}$   $\times$  2–6  $\mu\text{m}$  in size after fixation, and scattered in cytoplasm although most are clustered in mid-region of cell (Figures 1C,K). Micronucleus not observed. Often with 5–10 contractile vacuoles, 4–8  $\mu\text{m}$  in diameter, distributed along posterior 2/3 part of ventral margin (Figures 1A,H). Extrusomes thick bar-shaped, straight or slightly curved, about 10  $\mu\text{m}$  long, densely arranged along anterior part of buccal area, some scattered in cytoplasm (Figures 1B,C,I). Pellicle thin with small (<0.5  $\mu\text{m}$  across), densely spaced, grayish, dot-like cortical granules between ciliary rows on both sides of cell (Figures 1D,J). Cytoplasm colorless to pale yellow, often with numerous tiny, refringent globules (1–3  $\mu\text{m}$  across) that render main part of body opaque (Figures 1G,H). Locomotion usually by gliding

slowly on substrate, or swimming with a slow clockwise rotation about longitudinal axis.

Ciliary pattern as shown in Figures 1E,F,L,M. Eight to twelve left kineties (mean 9.9; median 10), including perioral kinety 1 and dorsal brush kinety (DB) which extends to about anterior 2/5 of cell-length and is composed of regularly spaced dikinetids (Figures 1E,L). Right side with 29–38 (mean 33.0; median 33) ciliated kineties including perioral kinety 2; intermediate somatic kineties are shortened forming a distinct anterior single-suture on right side (Figures 1F,M).

Two perioral kineties located along cytostome. Perioral kinety 1 (PK1) left of oral slit, composed of dikinetids in anterior 1/3 and monokinetids in posterior 2/3 (Figure 1E). Perioral kinety 2 (PK2) right of oral slit, consists of widely spaced dikinetids in anterior 1/3 part and continues posteriorly as a row of monokinetids (Figure 1E).

## ***Amphileptus shenzhenensis* sp. n.** (Tables 3, 4 and Figure 2)

### Zoobank Registration Number of Work

urn:lsid:zoobank.org:pub:DEE074BB-7B4F-46E7-8CA1-47820074ABBC

### ZooBank Registration Number of *A. shenzhenensis* sp. n.

urn:lsid:zoobank.org:act:53746C79-64F6-46D3-9E04-4662951D7CED

### Diagnosis

Body 125–250  $\mu\text{m}$   $\times$  40–50  $\mu\text{m}$  *in vivo*; slightly contractile with an inconspicuous beak-like anterior body end; numerous (> 200) macronuclear nodules; 6–8 left and 22–27 right kineties; several

(6–12) contractile vacuoles along ventral side of posterior 2/3 of cell; extrusomes thick rod-shaped, densely arranged along oral slit; dot-like cortical granules; brackish habitat.

### Etymology

Named after Shenzhen, where this species was first isolated.

### Type Locality and Ecological Features

Futian mangrove wetland (22° 38' N, 114° 06' E), Shenzhen, China. Water temperature 26°C, salinity 19.1‰, and pH 7.3.

### Type Slides

One protargol slide with the holotype specimen (circled in black ink) and several paratype specimens is deposited in the Laboratory of Protozoology, Ocean University of China (OUC), Qingdao, China, with registration number WL20110413-03.

**TABLE 2** | List of species associated with *Amphileptus/Hemiophrys* and their current names and status.

Taxon (basonym)	Current name	Present status	Data resource
<i>Amphileptus anser</i> sensu Ehrenberg, 1833	<i>Pseudomonilicaryon anser</i> (Mueller, 1773) Vd'ačný and Foissner, 2012	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus cygnus</i> Claparède and Lachmann, 1859	<i>Pseudomonilicaryon anser</i> (Mueller, 1773) Vd'ačný and Foissner, 2012	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus claparedii</i> Stein, 1867	<i>Apoamphileptus claparedii</i> (Stein, 1867) Lin and Song, 2004	Synonym	Lin and Song, 2004
<i>Amphileptus claparedei</i> sensu Bick (1972)	<i>Apoamphileptus claparedii</i> (Stein, 1867) Lin and Song, 2004	Synonym	Lin and Song, 2004
<i>Amphileptus claparedei</i> sensu Foissner et al. (1995)	<i>Apoamphileptus claparedii</i> (Stein, 1867) Lin and Song, 2004	Synonym	Lin and Song, 2004
<i>Amphileptus flagellatus</i> Rousslett, 1890	<i>Paradileptus elephantinus</i> (Švec, 1897) Kahl, 1931	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus gigas</i> Claparède and Lachmann, 1859	<i>Monomacrocaryon gigas</i> (Claparède and Lachmann, 1859) Vd'ačný et al., 2011	Reliable dileptid*	Vd'ačný and Foissner, 2012
<i>Amphileptus irregularis</i> Maskell, 1887	<i>Dileptus margaritifer</i> (Ehrenberg, 1833) Dujardin, 1841	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus lacazei</i> Gourret and Roeser, 1886	<i>Rimaleptus lacazei</i> (Gourret and Roeser, 1886) Vd'ačný and Foissner, 2012	Reliable dileptid*	Vd'ačný and Foissner, 2012
<i>Amphileptus longicollis</i> Ehrenberg, 1831	<i>Pseudomonilicaryon anser</i> (Mueller, 1773) Vd'ačný and Foissner, 2012	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus margaritifer</i> Ehrenberg, 1833	<i>Dileptus margaritifer</i> (Ehrenberg, 1833) Dujardin, 1841	Reliable dileptid*	Vd'ačný and Foissner, 2012
<i>Amphileptus massiliensis</i> Gourret and Roeser, 1886	–	Identity unclear	Vd'ačný and Foissner, 2012
<i>Amphileptus monilatus</i> Stokes, 1886	<i>Monilicaryon monilatum</i> (Stokes, 1886) Jankowski, 1967	Reliable dileptid*	Vd'ačný and Foissner, 2012
<i>Amphileptus moniliger</i> Ehrenberg, 1835	<i>Paradileptus elephantinus</i> (Švec, 1897) Kahl, 1931	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus ovum</i> sensu Dujardin, 1841	<i>Trachelius ovum</i> (Ehrenberg, 1831) Ehrenberg, 1833	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus vorax</i> sensu Dujardin, 1841	<i>Trachelius ovum</i> (Ehrenberg, 1831) Ehrenberg, 1833	Synonym	Vd'ačný and Foissner, 2012
<i>Amphileptus tracheloides</i> Maskell, 1887	–	Identity unclear	Vd'ačný and Foissner, 2012
<i>Amphileptus viridis</i> Ehrenberg, 1833	<i>Dileptus viridis</i> (Ehrenberg, 1833) Foissner, 1987	Reliable dileptid*	Vd'ačný and Foissner, 2012
<i>Amphileptus vareaeus</i> sensu Chen, 1955	<i>Amphileptus voracus</i> Davis, 1947	Synonym	Chen, 1955
<i>Hemiophrys macrostoma</i> Chen, 1955	<i>Pseudoamphileptus macrostoma</i> (Chen, 1955) Foissner, 1983	Synonym	Small and Lynn, 1981
<i>Hemiophrys lanceolatus</i> Dragesco, 1965	<i>Amphileptus lanceolatus</i> (Dragesco, 1965) Lin et al., 2005b	Synonym	Lin et al., 2005b
<i>Hemiophrys polymicronuclei</i> Li, 1990	<i>Amphileptus polymicronuclei</i> (Li, 1990) comb. nov.	Synonym	Li, 1990
<i>Hemiophrys salmicus</i> Burkovsky, 1970b	<i>Amphileptus salmicus</i> (Burkovsky, 1970b) comb. nov.	Synonym	Burkovsky, 1970b

\*Considered to be a reliable dileptid.

### SSU rDNA Sequence

The SSU rDNA sequence of *Amphileptus shenzhenensis* is deposited in the GenBank database with the accession number, length, and GC content as follows: MT653621, 1534 bp, 43.22%.

**TABLE 3** | Morphological characteristics of *Amphileptus multinucleatus* (1st line), *A. shenzhenensis* sp. n. (2nd line) and *A. cocous* sp. n. (3rd line).

Characters	Min	Max	Mean	SD	CV	n
Body length	200	450	296.7	12.64	4.3	24
	130	250	175.9	5.16	2.9	29
	175	400	284.3	14.70	5.2	20
Body width	60	120	79.0	3.14	4.0	24
	35	85	60.3	2.24	3.7	29
	45	190	93.4	10.80	11.6	20
Number of right Kineties <sup>a</sup>	29	38	33.0	0.65	2.0	20
Number of left Kineties <sup>b</sup>	22	27	24.0	0.29	1.2	29
	27	34	29.6	0.43	1.5	22
Length of Macronuclear Nodules	8	12	9.9	0.29	2.9	17
	6	8	6.9	0.13	1.8	29
Width of Macronuclear Nodules	7	10	8.6	0.20	2.4	22
	3	7	5.0	0.20	4.0	24
Length of Extrusomes	3	10	5.5	0.32	5.7	29
	3	10	6.7	0.46	6.9	20
Length of Extrusomes	2	6	4.0	0.25	6.2	24
	3	7	4.2	0.20	4.8	29
Length of Extrusomes	3	10	5.7	0.48	8.4	20
	8	12	9.1	0.22	2.4	24
Extrusomes	6	10	8.1	0.19	2.3	29
	7	11	9.0	0.20	2.2	22

All measurements in  $\mu\text{m}$ . Data based on protargol-stained specimens. CV, coefficient of variation in%; Max, maximum; Mean, arithmetic mean; Min, minimum; n, sample size; SD, standard deviation.

<sup>a</sup>Perioral kineties 2, 3 included.

<sup>b</sup>Perioral kinety 1 and dorsal brush kinety included.

### Morphological Description

Body 125–250  $\times$  40–50  $\mu\text{m}$  *in vivo*, usually 180–200 in length. Laterally compressed about 3:1, not flexible, with an inconspicuous, beak-like anterior end, a bluntly pointed posterior end, and a “neck” region about 1/5 of cell length (Figures 2A,G,H). Numerous macronuclear nodules (>200) scattered in cytoplasm but mostly clustered in central region of cell, about 5  $\mu\text{m}$  in diameter *in vivo*, usually discernable in life as slightly transparent areas (Figures 2F,M–O). Micronucleus not observed. Six–12 contractile vacuoles, about 5–15  $\mu\text{m}$  in diameter, located near ventral margin in posterior 2/3 of cell (Figures 2A,G,H,J). Extrusomes thick rod-shaped, straight to slightly curved, 6–8  $\mu\text{m}$  long, some evenly arranged along oral slit, others scattered in cytoplasm (Figures 2D,K,N,O). Pellicle thin with numerous small (< 0.5  $\mu\text{m}$  across), grayish, dot-like cortical granules, densely distributed between ciliary rows on both sides of cell (Figures 2E,I,L). Right side densely ciliated with cilia about 8  $\mu\text{m}$  long arranged in rows located within conspicuous longitudinal furrows, and forms a distinct anterior single-suture that is detectable *in vivo* (Figures 2G,H). Left somatic cilia generally sparsely distributed, dorsal brush cilia detectable at high magnifications, about 2–3  $\mu\text{m}$  long (Figure 2I). Cytoplasm slightly grayish, often with several food vacuoles, about 5–20  $\mu\text{m}$  in diameter (Figure 2J). Generally sensitive to disturbance, tending to form a resistant cyst after being placed on glass slide (Figure 2J). Locomotion by gliding moderately fast on substrate or by swimming while rotating clockwise about longitudinal axis.

Ciliary pattern as shown in Figures 2B,C,N–P. Left side with 6–8 (mean 6.9; median 7) ciliated kineties including perioral kinety 1 (PK1) and dorsal brush kinety (DB) which extends to about 2/5 of cell length and is composed of widely spaced dikinetids (Figures 2B,N,O). Twenty-two–27 (mean 24.0; median 24) right kineties including perioral kinety 2 (PK2); intermediate somatic kineties shortened forming a distinct suture in anterior part of body (Figures 2C,P).

**TABLE 4** | List of all known *Amphileptus* spp. of multiple macronuclear nodules ( $\geq 4$ ) and two new species in present study.

Species	LB ( $\mu\text{m}$ ) <sup>a</sup>	LK/RK <sup>b</sup> (median)	Body shape	S-CG <sup>d</sup>	N and p-CV <sup>e</sup>	Data source
<i>A. multinucleatus</i>	150–250	–/– <sup>c</sup>	Posterior with twist	–	6–10; posterior 2/3 of ventral	Wang, 1934
<i>A. multinucleatus</i>	250–450	8–12 (10)/29–38 (33)	Posterior with twist	Dot-like	5–10; posterior 2/3 of ventral	This study
<i>A. shenzhenensis</i> sp. n.	135–200	6–8 (7)/22–27 (24)	Rhombic	Dot-like	6–12; posterior 2/3 of ventral	This study
<i>A. cocous</i> sp. n.	180–350	7–10 (9)/27–34 (30)	Elongated blade-shaped	Rice-shaped	6–8; posterior 1/2 of ventral	This study
<i>A. quadrinucleatus</i>	400–650	–/30–34	Broad spindled	Dot-like	Many; both sides of cell	Dragesco and Dragesco-Kernéis, 1986
<i>A. aeschtae</i>	150–350	7–10 (9)/29–34 (31)	Ellipsoid	Dot-like	5–13; posterior 2/3 of ventral	Lin et al., 2007a

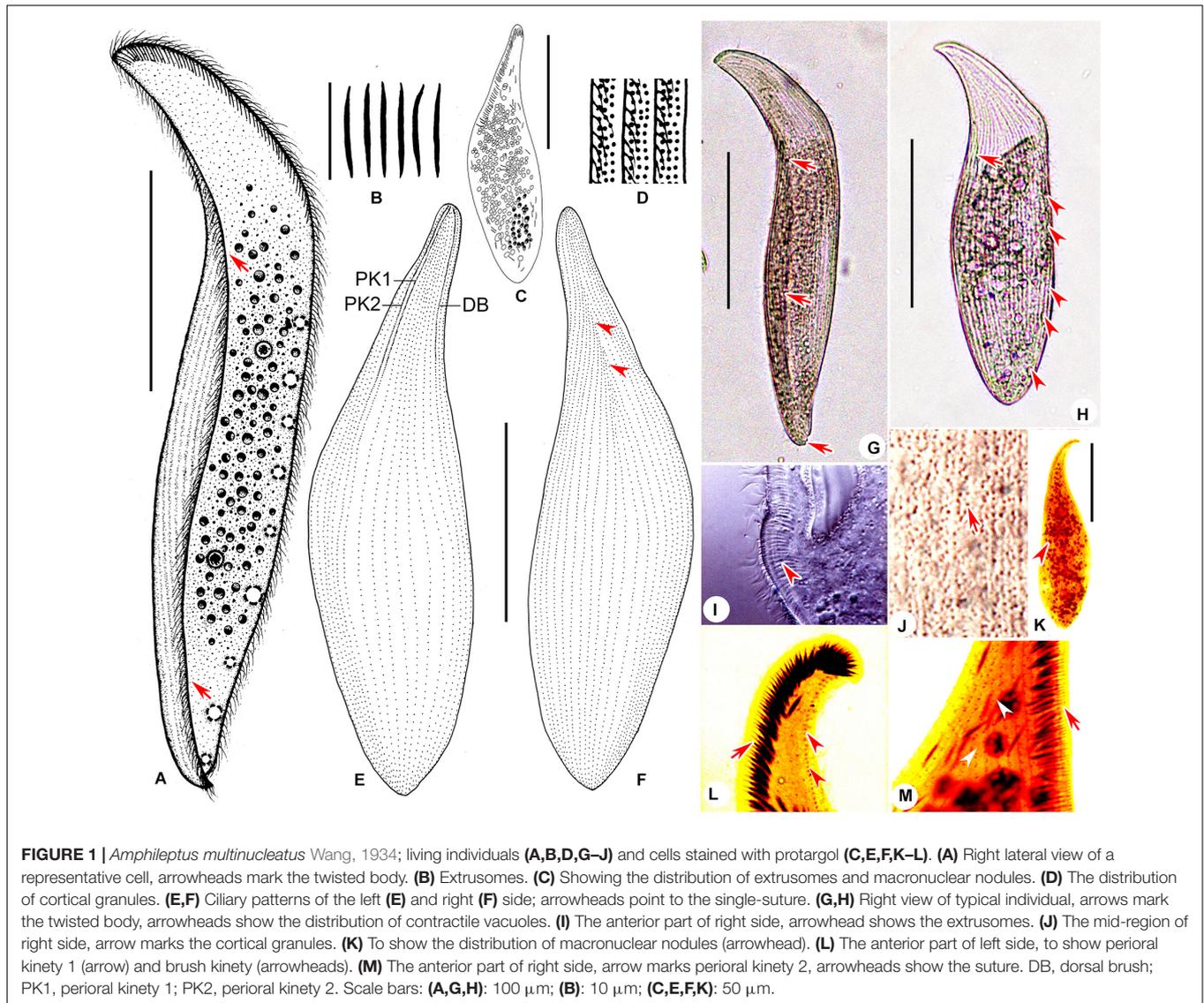
<sup>a</sup>Length of body *in vivo*.

<sup>b</sup>Number of left kineties/number of right kineties.

<sup>c</sup>Data not available.

<sup>d</sup>Shape of cortical granules.

<sup>e</sup>Number and position of contractile vacuoles.



**FIGURE 1** | *Amphileptus multinucleatus* Wang, 1934; living individuals (**A,B,D,G–J**) and cells stained with protargol (**C,E,F,K–L**). (**A**) Right lateral view of a representative cell, arrowheads mark the twisted body. (**B**) Extrusomes. (**C**) Showing the distribution of extrusomes and macronuclear nodules. (**D**) The distribution of cortical granules. (**E,F**) Ciliary patterns of the left (**E**) and right (**F**) side; arrowheads point to the single-suture. (**G,H**) Right view of typical individual, arrows mark the twisted body, arrowheads show the distribution of contractile vacuoles. (**I**) The anterior part of right side, arrowhead shows the extrusomes. (**J**) The mid-region of right side, arrow marks the cortical granules. (**K**) To show the distribution of macronuclear nodules (arrowhead). (**L**) The anterior part of left side, to show perial kinety 1 (arrow) and brush kinety (arrowheads). (**M**) The anterior part of right side, arrow marks perial kinety 2, arrowheads show the suture. DB, dorsal brush; PK1, perial kinety 1; PK2, perial kinety 2. Scale bars: (**A,G,H**): 100  $\mu\text{m}$ ; (**B**): 10  $\mu\text{m}$ ; (**C,E,F,K**): 50  $\mu\text{m}$ .

Two perial kineties along cytostome. Perial kinety 1 (PK1) left of oral slit, comprises dikinetids in anterior 2/5 and continues posteriorly as a row of monokinetids (**Figures 2B,N,O**). Perial kinety 2 (PK2) right of oral slit, comprises regularly spaced dikinetids in anterior 1/3 and monokinetids in posterior 2/3 (**Figures 2B,P**).

### ***Amphileptus cocous* sp. n. (Tables 3, 4 and Figure 3)**

ZooBank Registration Number of *Amphileptus cocous* sp. n.

urn:lsid:zoobank.org:act:96115FDB-E205-4FB0-ADE8-96DFC7767F60

#### **Diagnosis**

Medium to large *Amphileptus*, 180–350  $\times$  35–45  $\mu\text{m}$  *in vivo*; body strongly contractile, elongated blade-shaped; numerous (> 200) macronuclear nodules; 7–10 left and 27–34 right kineties;

several (6–8) contractile vacuoles along ventral side of posterior half of cell; extrusomes thick bar-shaped, densely arranged along oral slit; rice-shaped cortical granules; brackish habitat.

#### **Etymology**

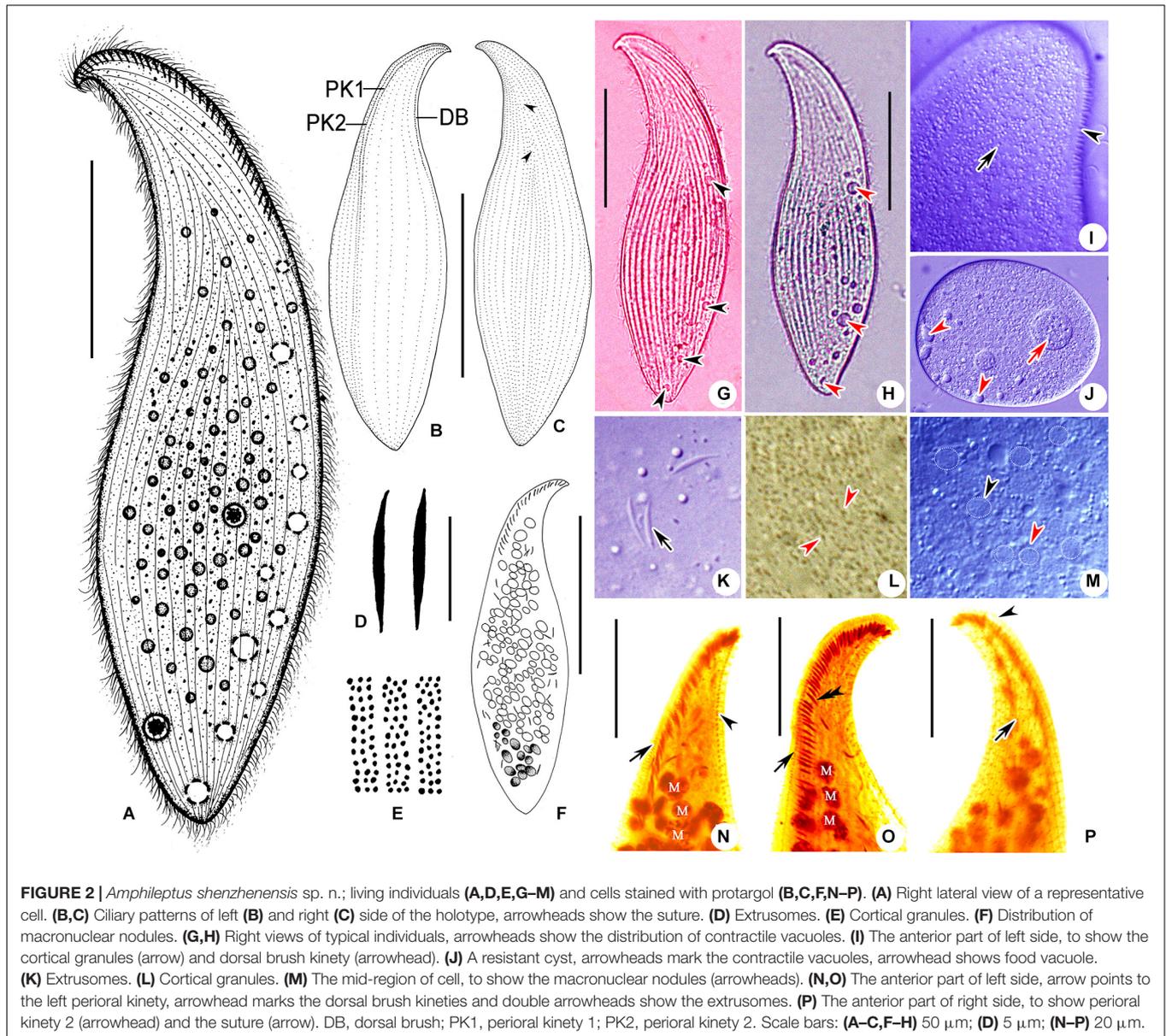
The Latin adjective “*cocous*” (rice-shaped) refers to the shape of cortical granules.

#### **Type Locality and Ecological Features**

Daya Bay mangrove wetland (22°41'N, 114°23'E), Huizhou, China. Water temperature 26.8°C, salinity 15.9‰, and pH 7.0.

#### **Type Slides**

One protargol slide with the holotype specimen (circled in black ink) and several paratype specimens is deposited in the Laboratory of Protozoology, Ocean University of China (OUC), Qingdao, China, with registration number WL20111027-01.



**FIGURE 2** | *Amphileptus shenzhenensis* sp. n.; living individuals (A,D,E,G-M) and cells stained with protargol (B,C,F,N-P). (A) Right lateral view of a representative cell. (B,C) Ciliary patterns of left (B) and right (C) side of the holotype, arrowheads show the suture. (D) Extrusomes. (E) Cortical granules. (F) Distribution of macronuclear nodules. (G,H) Right views of typical individuals, arrowheads show the distribution of contractile vacuoles. (I) The anterior part of left side, to show the cortical granules (arrow) and dorsal brush kinety (arrowhead). (J) A resistant cyst, arrowheads mark the contractile vacuoles, arrowhead shows food vacuole. (K) Extrusomes. (L) Cortical granules. (M) The mid-region of cell, to show the macronuclear nodules (arrowheads). (N,O) The anterior part of left side, arrow points to the left perial kinety, arrowhead marks the dorsal brush kineties and double arrowheads show the extrusomes. (P) The anterior part of right side, to show perial kinety 2 (arrowhead) and the suture (arrow). DB, dorsal brush; PK1, perial kinety 1; PK2, perial kinety 2. Scale bars: (A-C,F-H) 50 μm; (D) 5 μm; (N-P) 20 μm.

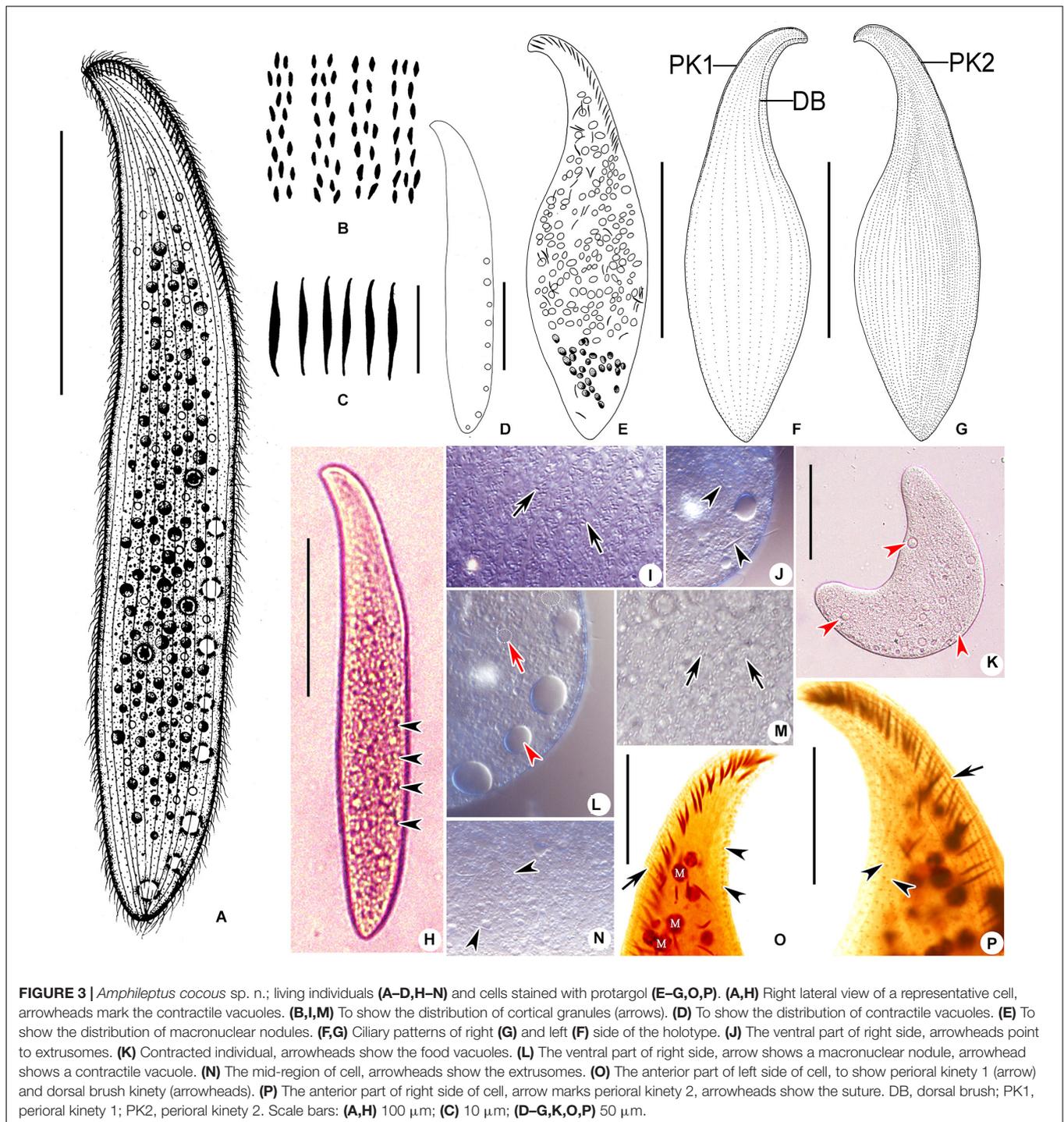
### SSU rDNA Sequence

The SSU rDNA sequence of *Amphileptus cocous* is deposited in the GenBank database with the accession number, length, and GC content as follows: MT653622, 1533 bp, 43.25%.

### Morphological Description

Body size highly variable, about 180–350 × 30–45 μm *in vivo*, usually 200–250 μm in length. Elongated blade-shaped, flexible, and strongly contractile, with widely pointed posterior end and an inconspicuous neck region about 15–20% of cell length and usually curved slightly to dorsal side, laterally compressed about 2–3:1 (Figures 3A,H). Numerous (>200) macronuclear nodules, ovoid to elliptical in outline, about 5–10 μm × 5–10 μm in size *in vivo*, scattered in cytoplasm, usually discernable in life as slightly

transparent areas (Figure 3L). Micronucleus not observed. Six–eight contractile vacuoles, about 8–13 μm in diameter, distributed along ventral margin in posterior half of cell (Figures 3A,D,H,L,K). Extrusomes spindle-shaped, about 10 μm long, some arranged in oral area, others some scattered in cytoplasm (Figures 3C,E,J,N,O). Pellicle thin with numerous short (about 1.0 μm in length), rice-shaped, colorless cortical granules densely packed between ciliary rows on both sides of cell (Figures 3B,I,M). Right side flat and densely ciliated, cilia about 8 μm long; left side sparsely ciliated, cilia difficult to detect in life. Cytoplasm colorless to grayish, often with numerous tiny, refringent globules (2–5 μm across) and numerous food vacuoles (3–10 μm across) that render main part of body opaque (Figures 3H,K). Locomotion moderately fast, usually gliding



on substrate or swimming with a slow clockwise rotation about longitudinal axis.

Ciliary pattern as shown in **Figures 3F, G, O, P**. About 7–10 (mean 8.6; median 9) widely spaced left kineties, including perial kinety 1 (PK1) and dorsal brush (DB) kinety which extends to 2/3 cell-length and is composed of regularly spaced dikinetids (**Figures 3F, O**). Right side densely ciliated, about 27–34 (mean 29.6; median 29) kineties including perial kinety

2, intermediate somatic kineties shortened anteriorly forming a distinct anterior suture (**Figures 3G, P**).

Two perial kineties around cytostome: PK1 left of oral slit, composed of closely spaced dikinetids in anterior 2/5 and continues posteriorly as a row of closely spaced monokinetids (**Figures 3F, O**); PK2 right of oral slit, formed of closely spaced dikinetids in anterior 2/5 and continues posteriorly as a row of closely spaced monokinetids (**Figures 3G, P**).



with *A. dragescoi* (81% MP) to form the second clade (clade II in **Figure 4B**), and the remaining two species (*Pseudoamphileptus macrostoma* and *Amphileptus* sp.) form the third clade (clade III in **Figure 4B**) with high support (99% MP).

## DISCUSSION

### A Brief Summary of the Genus *Amphileptus* Ehrenberg, 1830

The genus *Amphileptus* was established by Ehrenberg (1830) and another pleurostomatid genus, *Hemiophrys*, was established by Wrzesniowski (1870). Until the mid-twentieth century, descriptions of *Amphileptus-Hemiophrys* species were exclusively based on observations of live species (Ehrenberg, 1830; Kahl, 1931). Canella (1960) carried out the first detailed investigation of the ciliary pattern of these two genera using silver staining and revealed that the somatic kineties on the right side form a suture in the mid-to-anterior region of the cell in both genera. Furthermore, other morphological differences were regarded as species-level rather than genus-level characters, suggesting that *Hemiophrys* is a junior synonym of *Amphileptus* (Canella, 1960). This recommendation was accepted by Fryd-Versavel et al. (1975). Consequently, the genus *Hemiophrys* was merged into the genus *Amphileptus*, and the diagnosis of genus *Amphileptus* was emended, i.e., the formation of a single suture by the somatic kineties of the right side was considered to be the key diagnostic character. The emended diagnosis of the genus *Amphileptus* and the submersion of *Hemiophrys* were accepted by subsequent investigators (Foissner, 1984; Aescht, 2001; Lynn, 2008).

Species of the genus *Amphileptus* are easily identified by the pattern of right somatic kineties, i.e., the somatic kineties shortened to form a single suture in the median area, and this is the main differentiating feature for certain pleurostomatid genera, e.g., *Amphileptus* and *Apoamphileptus* within the order Pleurostomatida. Therefore, the presence of a single suture is thought to be an important genus-level, and possibly even a family-level character (Lin et al., 2005b; Wu et al., 2017).

The ciliature of *Amphileptus* consists of perial kineties, right somatic kineties, left somatic kineties and dorsal brush kineties, the number and pattern of which are important for species delimitation. The key characteristics for species determination are: (1) body shape and size; (2) number of right and/or left kineties; (3) number and position of contractile vacuoles; (4) number of macronuclear nodules; and (5) presence vs. absence and shape of cortical granules (Canella, 1960; Foissner, 1984; Song, 1991; Lin et al., 2007a; Wu et al., 2015b).

The first molecular phylogenetic study of *Amphileptus* was that of Gao et al. (2008) who sequenced the SSU rDNA of *A. procerus* (Penard, 1922) Song and Wilbert, 1989 and *A. aeschtae* Lin et al., 2007a. Pan et al. (2014) added another new sequence and reported the monophyly of the family Amphileptidae and the paraphyly of the genus *Amphileptus* with *Pseudoamphileptus* nested within it. These findings have been both confirmed and rejected in subsequent studies (Wu et al., 2015b, 2017; Pan et al., 2015) (see molecular analyses below). Including the three species described in the present study, there

are nine identified and one unidentified SSU rDNA sequences in the NCBI/GenBank database.

### Comments on *Amphileptus multinucleatus* Wang, 1934

*Amphileptus multinucleatus* was originally described by Wang (1934) who gave a good description based on live observations, although the ciliary pattern was not mentioned. It was characterized mainly as follows: body 150–250  $\mu\text{m}$  in length, with 6–10 contractile vacuoles lying in ventral posterior half, numerous (40–70) macronuclear nodules scattered in cytoplasm, and posterior end twisted to one side. In particular, it was noted that “the twisted posterior portion is very constant in all observed individuals and may be considered as one of the specific characteristics” (Wang, 1934). This specific feature was also observed in all observed individuals of the Daya Bay population ( $n > 20$ ) and has not been recorded in any other species of *Amphileptus*. We conclude that the twisted posterior portion of the body should be considered as a diagnostic character of this species. In addition, our form was identified as *A. multinucleatus* based on the distribution of extrusomes along the oral slit and with some scattered in the cytoplasm. One significant difference between the original description and the Daya Bay population of *A. multinucleatus* is the number of macronuclear nodules, the former having 40–70 and the latter 80–300. It should be noted, however, that the number of macronuclear nodules in the original description was based on observations of specimens fixed in Schaudinn’s fluid and stained with iron-alum-hematoxylin which may not show the outline of the macronuclear nodules as clearly as the protargol stain. In addition, the body size of the Daya Bay population is considerably larger than that of the original population (200–400 vs. 150–250  $\mu\text{m}$  in length), which may be another reason for the higher number of macronuclear nodules. We therefore conclude the Daya Bay population is conspecific with the original population of *A. multinucleatus* described by Wang (1934).

### Comments on *Amphileptus shenzhenensis* sp. n. and *A. cocous* sp. n.

The most important characters for species identification and circumscription in the genus *Amphileptus* include the number of kineties, the number and positions of contractile vacuoles, the number of macronuclear nodules, the shape and distribution of extrusomes, the presence or absence and the shape of cortical granules, and the body shape *in vivo* (Foissner et al., 1995; Lin and Song, 2004; this study).

Among all the nominal species of *Amphileptus*, only three congeners are reported to have contractile vacuoles arranged along the ventral margin of the cell and four or more macronuclear nodules, i.e., *A. multinucleatus*, *A. quadrinucleatus* and *A. aeschtae* (Wang, 1934; Dragesco and Dragesco-Kernéis, 1986; Lin et al., 2007a; **Table 4**). The two new forms and the three described species strongly resemble each other in body size, position and number of macronuclear nodules, shape of cortical granules, and the number of kineties. However, *A. multinucleatus*

is the only species in this genus with a posterior end constantly twisted to one side (Wang, 1934), therefore it can be clearly distinguished from the other species.

*Amphileptus shenzhenensis* sp. n. resembles *A. multinucleatus*, *A. quadrinucleatus*, and *A. aeschtae* in having dot-like cortical granules. However, *A. shenzhenensis* sp. n. differs: from *A. multinucleatus* by having fewer kineties on both sides of the cell (6–8 vs. 8–12 on left; 22–27 vs. 29–38 on right), and the posterior portion of the body not twisted (Wang, 1934); from *A. quadrinucleatus* by having fewer right kineties (22–27 vs. 30–34), the distribution of contractile vacuoles (in posterior 2/3 of cell on ventral side vs. down the length of both sides of body) and its significantly smaller body length (135–200 vs. 400–650  $\mu\text{m}$  *in vivo*) (Dragesco and Dragesco-Kernéis, 1986); from *A. aeschtae* by having fewer kineties on both sides of cell, i.e., 6–8, mean 7 vs. 7–10, mean 9 on left; 22–27 vs. 29–34 on right (Lin et al., 2007a; **Table 4**).

*Amphileptus cocous* sp. n. resembles *A. aeschtae* in having the same number of left kineties (7–9) and almost the same number of right kineties (27–34, mean 30 vs. 29–34, mean 31). However, the former can be separated from the latter by the different shape of cortical granules (rice-shaped vs. dot-like), the distribution of contractile vacuoles (in the posterior half of the body vs. in the posterior 2/3 of the body) and the elongated blade-shaped (vs. ellipsoidal) body (Lin et al., 2007a; **Table 4**).

*Amphileptus shenzhenensis* sp. n. can be distinguished from *A. cocous* sp. n. by having fewer right kineties (22–27, mean 24 vs. 27–34, mean 30) and dot-like (vs. rice-shaped) cortical granules (**Table 4**).

The phylogenetic analyses based on SSU rDNA sequence data show that *Amphileptus cocous* sp. n. groups with *A. spiculatus* in the core clade. However, the former can be distinguished from the latter by the following combination of morphological characters: (1) the different body shape *in vivo* (elongated blade-shaped vs. pyriform); (2) the rice-shaped (vs. dot-like) cortical granules; (3) the number of right kineties (27–34 vs. 11–14); (4) the larger body size (180–350 vs. 85–145  $\mu\text{m}$  long *in vivo*), and (5) having significantly more (>300 vs. 2) macronuclear nodules (Wu et al., 2015b). In addition, the validity of the new species is supported by the SSU rDNA sequence data: *Amphileptus cocous* sp. n. differs in one, two, and five nucleotides from *A. shenzhenensis* sp. n., *A. multinucleatus* and *A. aeschtae*, respectively; and *A. shenzhenensis* sp. n. differs in six and three nucleotides from *A. aeschtae* and *A. multinucleatus*, respectively.

## Two New Combinations of the Genus *Amphileptus*

Li (1990) reported a new species from Donghu Lake, Hubei Province, China, under the name *Hemiophrys polymicronuclei*. However, because *Hemiophrys* is a junior synonym of *Amphileptus*, this species should be transferred to the latter (Canella, 1960). Therefore, we propose a new combination, *Amphileptus polymicronuclei* (Li, 1990) comb. n. (original combination *Hemiophrys polymicronuclei* Li, 1990; **Tables 1, 2**). In addition, another species, *Hemiophrys salimica*, was reported

**TABLE 5** | Kishino-Hasegawa (KH) test results.

Topology constraints	lnL	KH value (P)
Best ML tree (unconstrained)	7582.22678250	0.996
Monophyly of <i>Amphileptus</i>	7615.42903834	0.004

$P < 0.05$  refutes monophyly;  $P > 0.05$  does not refute the possibility of monophyly.

by Burkovsky (1970b) from Kandalaksha Gulf, White Sea, which should be transferred to *Amphileptus* (Canella, 1960; Curds, 1982). Hence, a new combination, *Amphileptus salimicus* (Burkovsky, 1970b) comb. n. (original combination *Hemiophrys salimica* Burkovsky, 1970b), is suggested (**Tables 1, 2**).

## Comments on the Phylogeny of the Genus *Amphileptus*

The family Amphileptidae is characterized by the presence of a single anterior suture on the right side, thereby differentiating it from the other three families within the order Pleurostomatida (Vd'áčný et al., 2015; Wu et al., 2017). The Amphileptidae, comprises five genera, namely *Amphileptus* (the type genus), *Pseudoamphileptus*, *Amphileptiscus*, *Apoamphileptus*, and *Opisthodon*, but molecular data are available only the former two genera. Traditionally, the genus *Amphileptus* has been considered to be monophyletic based on morphological (Foissner, 1977, 1983, 1984; Corliss, 1979; Lin et al., 2005a,b, 2007a; Lynn, 2008) and some molecular studies (Pan et al., 2010, 2013; Zhang et al., 2012; Vd'áčný and Foissner, 2013; Wu et al., 2013, 2015b, 2017; Vd'áčný, 2015; Vd'áčný et al., 2015). However, the monophyly of this *Amphileptus* has been questioned by several other molecular phylogenetic studies (Gao et al., 2008; Vd'áčný et al., 2011, 2015; Pan et al., 2014, 2015; Wu et al., 2014, 2015b). In our SSU rDNA trees (**Figure 4**) which include three new sequences of *Amphileptus*, the non-monophyly of the genus *Amphileptus* was supported, since it was divided into two and three groups in ML/BI and MP tree, respectively. Furthermore, the possibility of the monophyly of *Amphileptus* was also rejected ( $p = 0.004 < 0.05$ ) by the KH test (**Table 5**). Although some previous studies have shown members of the genus *Amphileptus* to group together in phylogenetic trees (Pan et al., 2010, 2013; Zhang et al., 2012; Vd'áčný and Foissner, 2013; Wu et al., 2013, 2015a, 2017; Vd'áčný, 2015; Vd'áčný et al., 2015), these convergent topologies are based on insufficient taxon sampling and have only low nodal support (e.g., 76% ML, 0.65 BI in Pan et al., 2013; 17% ML, 20% MP in Wu et al., 2015a; 50% ML, 0.64 BI, 57% MP in Vd'áčný et al., 2015; 51% ML, 0.78 BI, 67% MP in Vd'áčný, 2015). Therefore, the molecular data strongly question the morphology-based relationship of the genus *Amphileptus*, and even the family Amphileptidae, and indicate that this group is paraphyletic. To date, however, only two genera of the family Amphileptidae have molecular data, namely *Amphileptus* and *Pseudoamphileptus*. It is noteworthy that *Pseudoamphileptus* is represented by a single sequence that clusters with sequences of *Amphileptus* (**Figure 4**). Furthermore, no morphological characters can be identified as plesiomorphic or apomorphic, so the question of whether the genus *Amphileptus* and/or the family Amphileptidae is monophyletic will remain unresolved pending

the availability of more morphological and molecular data with expanded taxon sampling.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GenBank (accession: MT653621, MT653622, and MT653624).

## AUTHOR CONTRIBUTIONS

LW and XL conceived and designed the manuscript. LW carried out the live observation and protargol staining. LW, JL, AW, and XL wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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