

# Morphology and molecular phylogeny of two colepid species from China, *Coleps amphacanthus* Ehrenberg, 1833 and *Levicoles biwae jejuensis* Chen et al., 2016 (Ciliophora, Prostomatida)

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## ABSTRACT

Two colepid ciliates, *Coleps amphacanthus* Ehrenberg, 1833 and *Levicoles biwae jejuensis* Chen et al., 2016, were first recorded in China. Their living morphology, infraciliature and small subunit (SSU) rRNA gene sequences were determined using standard methods. The improved diagnosis of *Coleps amphacanthus* is as follows: cell size about 100×50 μm *in vivo*, barrel-shaped; 22-28 ciliary rows each composed of about 14-21 monokinetids and two perioral dikinetids; 5-10 caudal cilia; and one terminal contractile vacuole. *Levicoles biwae jejuensis* was also investigated, with an improved diagnosis given based on previous and present work. The phylogenetic analyses based on SSU rRNA gene sequences revealed that all *Coleps* species were grouped together, except for *Coleps amphacanthus*, which was grouped into a clade of the genus *Levicoles*.

**Keywords:** Ciliate; *Coleps*; *Levicoles*; New record; Phylogeny; SSU rRNA; Taxonomy

## INTRODUCTION

Colepid ciliates are commonly found in a wide range of habitats, including benthic, pelagic and marine psammobiotic. They are one of the main components of the microbial community and play an important role in the function of the microbial food webs (Finlay & Fenchel, 1996; Song et al., 2009). Since the first species, *Coleps hirtus* (Müller, 1786) Nitzsch, 1827, was reported two centuries ago, more than 40 species of this family have been found and recorded. They are characterized by cylindrical or barrel-shaped bodies covered by unique calcified cuirasses (armored plates with small lateral plate processes arranged in longitudinal rows), sometimes with anterior and/or posterior spines. In general, their caudal cilia are clearly longer

than other somatic cilia (Corliss, 1979; Dragesco & Dragesco-Kernéis, 1991; Foissner, 1983; Kahl, 1930; Noland, 1925; Obolkina, 1995). Individual taxa may be characterized by a variety of morphological features including the number of armor tiers, structure of plates, presence or absence of spines, and number of adoral organelles (Foissner et al., 2008). According to these taxonomic standards, some new genera have been established in recent years, including the construction of *Levicoles* due to the absence of spines and armor tiers with *hirtus*-type plates and *Kotinia* characterized by spiny armor composed of eight tiers and five adoral organelles (Chen et al., 2009, 2010, 2012, 2016; Foissner et al., 2008; Obolkina, 1995).

The present paper provides a morphological description of a poorly known species, *Coleps amphacanthus* Ehrenberg, 1833, and a new record of *Levicoles biwae jejuensis* Chen et al., 2016, in China. Phylogenetic analyses based on SSU rRNA gene sequences were also performed.

## MATERIALS AND METHODS

### Sample collection, observation, and identification

*Coleps amphacanthus* Ehrenberg, 1833 was collected from the brackish water of Hangzhou Bay, Ningbo (N30°22'; E121°12'), China, on 22 July, 2014. The water temperature was about 25°C and salinity was about 2‰. Samples with decaying plants were collected from the surface of the sediment using a plastic dropper, then diluted with untreated water from the collection site.

*Levicoles biwae jejuensis* Chen et al., 2016 was collected on 22 June, 2015, from a freshwater pond in Ningbo (N29°55';

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E121°39'). The water temperature was about 23 °C. Samples were collected by 20 µm mesh plankton nets from the upper layer of the water.

The behavior of the organisms was studied in Petri dishes under a dissecting microscope. Living morphology was investigated under a compound microscope equipped with a high-power oil immersion objective as well as differential interference contrast optics (Olympus BH-2, Olympus Optical Co., Ltd, Tokyo, Japan). The infraciliature was revealed via protargol impregnation (Wilbert, 1975). Counts and measurements of the morphological characteristics of stained specimens were performed at a magnification of ×1 250. Drawings were made with the help of a camera lucida. Terminology used is primarily in accordance with Foissner et al. (2008) and Chen et al. (2010).

#### DNA extraction, gene amplification, and sequencing

Genomic DNA was extracted from cells using the Dneasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The PCR amplification of the SSU rRNA gene sequences was performed using universal eukaryotic primers 18S-F (5'-AACCTGGTTGATCCTGCCAGT-3') and 18S-R (5'-TGATCCTTCTGCAGGTTACCTAC-3') (Medlin et al., 1988). PCR products were purified using an EasyPure® Quik Gel Extraction Kit (TransGen, EG101, Beijing, China), then cloned using a pEASY®-T1 Cloning kit (TransGen, CT101, Beijing, China). Sequencing was performed bidirectionally on an ABI 3700 sequencer (GENEWIZ Biotechnology Co., Ltd., Beijing, China).

#### Phylogenetic analyses

The two newly characterized SSU rRNA gene sequences and the sequences of another 45 species/populations obtained from the NCBI GenBank database were used for phylogenetic analyses. Sequences were aligned using Bioedit v7.1.3.0. (Hall, 1999) with the ClustalW algorithm. The resulting alignments were manually refined by trimming both ends. Final alignment included 1 767 characters and 47 taxa. *Paramecium tetraurelia*, *Ichthyophthirius multifiliis*, and *Tetrahymena pyriformis* were selected as outgroup taxa for phylogenetic analyses. Bayesian inference (BI) and maximum likelihood (ML) analyses were performed online using the CIPRES Science Gateway v3.3 (<http://www.phylo.org/portal2>). BI analysis was performed with MrBayes on XSEDE v3.2.6 (Ronquist & Huelsenbeck, 2003) using the GTR+I+G model as selected by MrModeltest v2.2 (Nylander, 2004). The chain length of the Bayesian analyses was 10 000 000 generations, sampled every 100 generations. The first 10 000 sampled trees were considered as burn-in. ML analysis was performed with RAxML-HPC2 on XSEDE v8.2.4 (Stamatakis et al., 2008) using the GTR+I+G model as selected by Modeltest v3.4 (Posada & Crandall, 1998). The reliability of ML internal branches was assessed using a nonparametric bootstrap method with 1 000 replicates. MEGA v5.0 (Tamura et al., 2011) was used to view and edit tree topologies. Systematic classification mainly followed Lynn (2008) and Yi et al. (2010).

#### Hypothesis testing

To test the hypothesis that the morphologically-defined genera

are monophyletic groups, RAxML (Shimodaira, 2002; Stamatakis et al., 2008) was used to generate ML trees with enforced topological constraints. For all constraints, internal relationships within the constrained groups and among the remaining taxa were unspecified. The site-wise likelihoods for the resulting constrained topologies and the non-constrained ML topologies were calculated using PAUP (Swofford, 2002). Approximately unbiased (AU) tests (Shimodaira, 2002) were performed using CONSEL v 0.1 (Shimodaira & Hasegawa, 2001) to obtain *P*-values.

## RESULTS

### *Coleps amphacanthus* Ehrenberg, 1833 (Table 1; Figure 1 and Figure 2)

*Coleps amphacanthus* is a poorly known and rarely reported species (Foissner & O'Donoghue, 1990; Huttenlauch, 1986, 1987; Huttenlauch & Bardele, 1987; Kreutz & Foissner, 2006; Noland, 1925). According to these historic studies, the morphological features are not particularly stable and most descriptions lack the infraciliature. Therefore, re-description is necessary to reduce confusion and clarify intraspecific variations.

**Improved diagnosis.** Cells about (80-110) µm×(40-60) µm *in vivo*, barrel shaped. On average 22-28 ciliary rows, each composed of about 14-21 monokinetids and two perioral dikinetids. Anterior and posterior main plates each with 5-8 and 5-6 windows, respectively; anterior and posterior secondary plates with 2-3 windows, respectively. Five to ten caudal cilia. One subterminal contractile vacuole. Fresh- or brackish-habitat.

**Voucher slide.** A voucher slide with protargol-impregnated specimens was deposited in the Laboratory of Protozoology, Ocean University of China (registration number: LU-20140722-04-01).

#### Morphological description based on Chinese population.

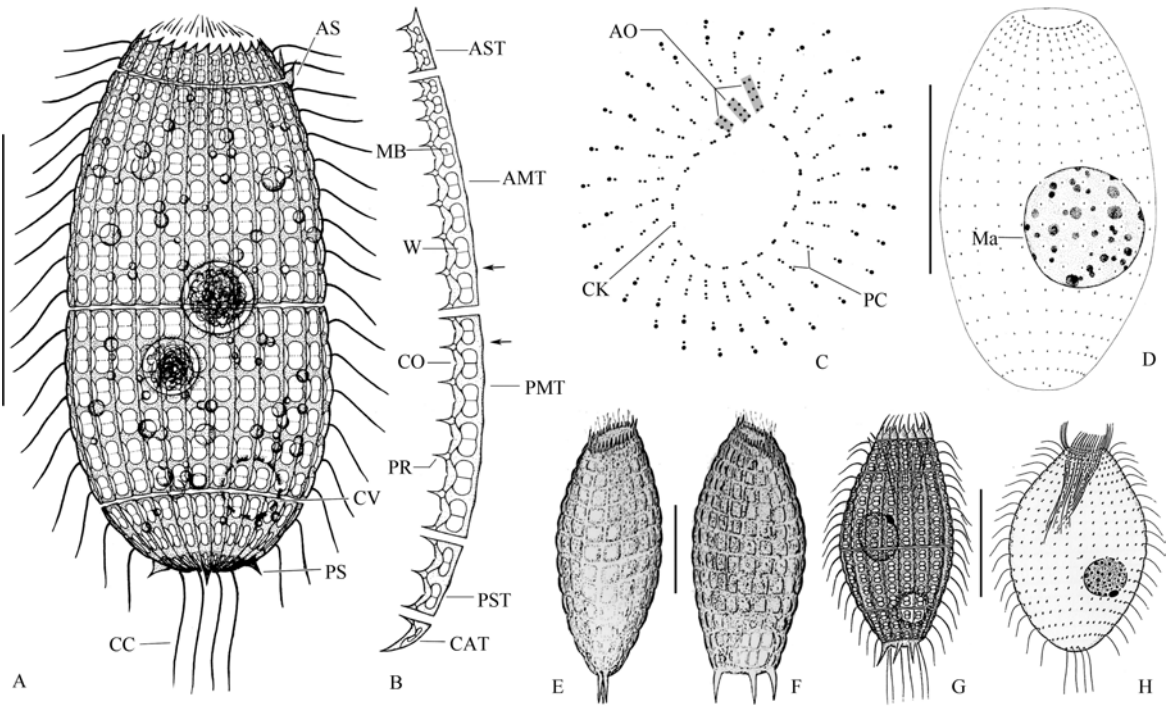
Body size (90-110) µm×(40-60) µm *in vivo*, usually about 100 µm×50 µm, barrel-shaped, with a slight wave-like arch at each window caused by sieve domes, usually slightly narrowed in the mid-body where the main armor plates abut. Ratio of body length to width about 2: 1. Anterior end broad, transversely truncated and crown-like due to triangle-shaped secondary tier plates; posterior end moderately rounded (Figure 1A; Figure 2A). Macronucleus spherical to broadly ellipsoidal with an average length: width ratio of 1.3, usually located at the equatorial level and near the periphery of the cell. Micronucleus globular and approximately 2 µm in diameter, adherent to the macronucleus (Figure 1D; Figure 2I, J). Contractile vacuole about 12 µm in diameter, positioned near the subterminal of the body (Figure 1A; Figure 2A). Cytoplasm colorless, usually containing several food vacuoles (10-20 µm in diameter) and mass of refractive lipid droplets (1-4 µm across) (Figure 1A; Figure 2A).

Armor composed of *hirtus*-type plates arranged in six tiers: circumoral, anterior secondary, anterior main, posterior main, posterior secondary, and caudal, with each tier consisting of about 25 rectangular plates (Figure 1A, B; Figure 2A). Circumoral tiers hardly recognizable *in vivo*. Anterior secondary plates about 12 µm in length, with two windows, two ciliary outlets,

**Table 1** Morphometric data on *Coleps amphacanthus* (first line) and *Levicoleps biwae jejuensis* (second line)

Characters	Min	Max	M	Mean	SD	SE	CV	n
Body length	82	98	90.0	89.0	4.9	2.0	5.5	8
	85	103	94.0	95.1	5.2	1.3	5.5	15
Body width	39	51	45.0	46.0	5.0	2.0	10.1	8
	37	57	47.0	45.7	6.6	1.7	14.4	15
Somatic kineties, number	24	26	25.0	25.0	0.8	0.0	3.2	8
	21	24	22.5	23.1	0.8	0.2	3.5	15
Transverse ciliary rows, number*	18	18	18.0	18.0	0.0	0.0	0.0	9
	15	15	15.0	15.0	0.0	0.0	0.0	15
Macronucleus length	25	32	28.5	29.3	2.6	0.9	8.9	8
	21	38	29.5	27.7	4.8	1.2	17.3	15
Macronucleus width	16	29	22.5	23.8	4.5	1.6	18.9	8
	17	32	24.5	21.2	3.9	1.0	18.4	15

Data based on protargol-impregnated specimens. All measurements in  $\mu\text{m}$ . CV: coefficient of variation in %, Max: maximum, Mean: arithmetic mean, M: median, Min: minimum, n: number of specimens analysed, SD: standard deviation, SE: standard error. \*: Excluding perioral dikinetids.



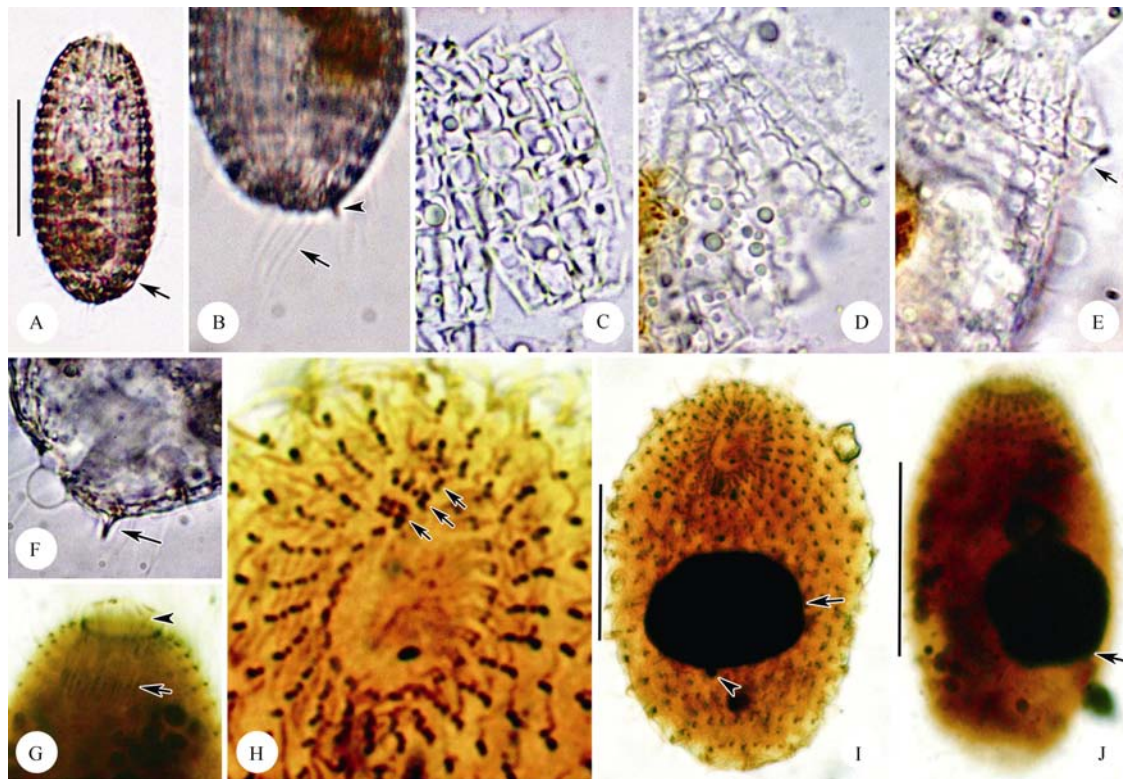
**Figure 1** Morphology and infraciliature of *Coleps amphacanthus*

A: Lateral view shows body shape, smallish spines and characters of plates. B: Detailed structure of row of plates. C: Apical view of infraciliature, showing the ciliary pattern of the top end. D: Lateral view of the infraciliature. E, F: Lateral views of *Coleps amphacanthus* (from Noland 1925). G, H: Lateral views of living individual (G) and infraciliature (H) (from Foissner & O'Donoghue, 1990). Abbreviations: AO, adoral organelle; AMT, anterior main tier; AS, anterior spine; AST, anterior secondary tier; CAT, caudal tier; CC, caudal cilia; CK, circumoral kinety; CO, ciliary outlet; CV, contractile vacuole; Ma, macronucleus; MB, midbar; PC, perioral ciliature; PMT, posterior main tier; PR, plate processes; PS, posterior spine; PST, posterior secondary tier; W, plate windows. Scale bars=50  $\mu\text{m}$  (A, D), 30  $\mu\text{m}$  (E, F), 40  $\mu\text{m}$  (G, H).

three plate processes, and triangle-shaped anterior end; usually two of them with anterior spines located at the same side (Figure 1A, B; Figure 2E). Anterior main plates approximately 40  $\mu\text{m}$  long, with eight windows gradually enlarged from anterior to posterior, nine plate processes, and eight ciliary outlets (Figure 1B; Figure 2D). Posterior main plates approximately 30  $\mu\text{m}$  long, with six windows, seven plate processes, and six ciliary outlets (Figure 1B; Figure 2C). Posterior secondary plates trapezoidal, about 12  $\mu\text{m}$  long, with two elongated windows, three plate processes, and two ciliary outlets (Figure 1B). Caudal tier visible only in oblique or posterior polar view, about 5  $\mu\text{m}$  long, usually with three small and sharp posterior spines (Figure 1A, B; Figure 2F). The fine structure of the armor plates is shown in Figure 1B; 2C-D: left

margin generally smooth and slightly convex at the level of the bridge; sharp plate processes arranged alternately with windows on the right edge, connected with conspicuous bridges; windows "8" shaped with small radian sieve domes, separated in pairs by a midbar; generally, 2, 8, 6, 2 windows in the middle four tiers from the anterior to posterior (Figure 1A; Figure 2A).

Oral opening at anterior end of cell. Circumoral kinety circular, composed of dikinetids, interrupted at the site of three adoral organelles. Adoral organelles short and obliquely arranged. Organelle 1 composed of three pairs of kinetosomes, organelles 2 and 3 each composed of four pairs of kinetosomes (Figure 1C; Figure 2H). Internal and external oral basket in center of mouth, about 6  $\mu\text{m}$  and 10  $\mu\text{m}$  long, respectively, conspicuous in protargol-impregnated specimens (Figure 2G).



**Figure 2** Microphotographs of living *Coleps amphacanthus* and after protargol impregnation

A: Lateral view of a typical individual, arrow indicates contractile vacuole. B: Lateral view of posterior section of the body, arrow marks caudal cilia, arrowhead denotes posterior spine. C: Posterior main plates. D: Anterior main plates. E: Lateral view of the anterior section of the cell, showing anterior spine (arrow). F: Lateral view of the posterior end of the body, arrow indicates posterior spine. G: Lateral view of anterior portion, showing internal (arrowhead) and external (arrow) oral basket. H: Detailed infraciliature of the apical end, arrows denote three adoral organelles. I: Infraciliature of a pressed specimen showing ciliary pattern, arrow denotes macronucleus, arrowhead indicates micronucleus. J: Lateral view of a specimen on a permanent slide, arrow denotes macronucleus. Scale bars=50  $\mu\text{m}$ .

Somatic cilia about 15  $\mu\text{m}$  long and regularly arranged, forming 18 transverse circles and 25 longitudinal rows on average (Figure 1D; Figure 2I, J). Anterior two transverse circles with dikinetids closely arranged and forming perioral ciliature (Figure 1C; Figure 2H). Five to ten significantly long caudal cilia (about 25  $\mu\text{m}$ ) (Figure 1A; Figure 2B).

#### ***Levicolaps biwae jejuensis* Chen et al., 2016 (Table 1; Figure 3 and 4)**

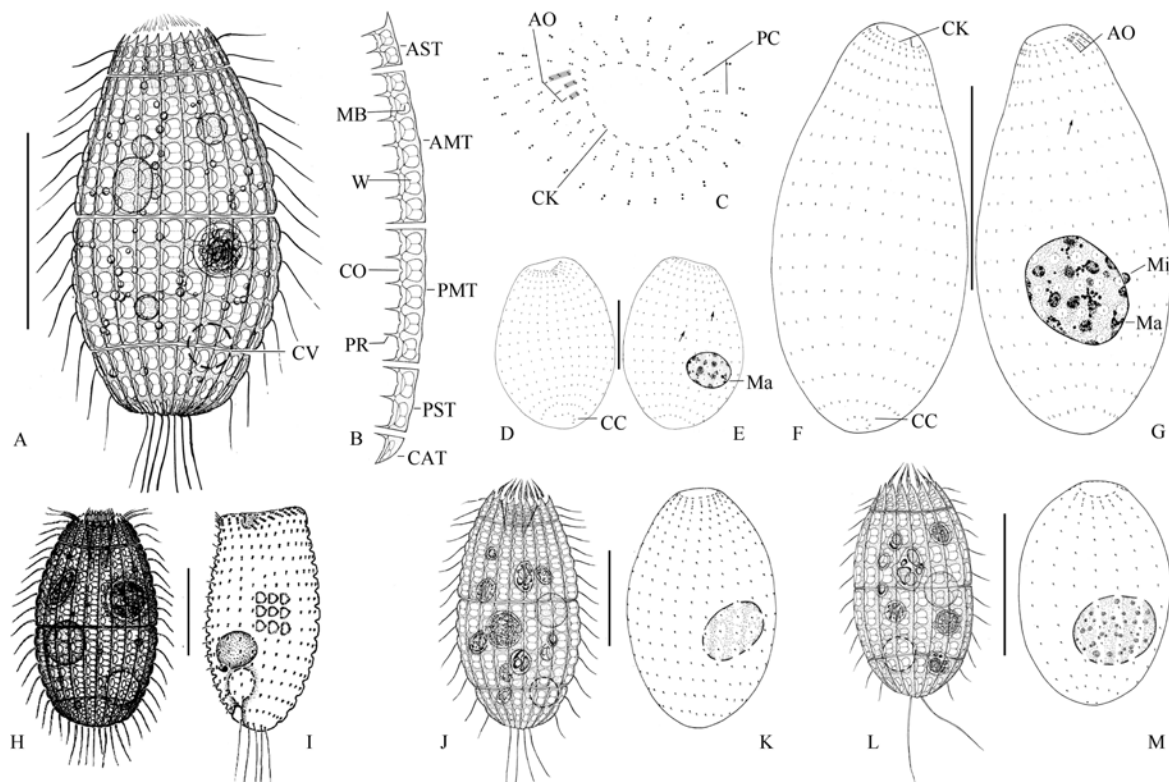
The genus *Levicolaps* was established by Foissner et al. (2008) with *Levicolaps biwae* as the type species. Chen et al. (2016) reported a South Korean subspecies, *L. biwae jejuensis*, from

Jeju Island. These two subspecies are very similar in morphological characters and infraciliature, except the number of caudal cilia. A Chinese population of *L. biwae jejuensis* is described here to clarify intraspecific variations.

**Improved diagnosis.** Size *in vivo* about (70–110)  $\mu\text{m}$   $\times$  (30–60)  $\mu\text{m}$ , barrel shaped. On average 21–24 ciliary rows, each composed of about 15–18 monokinetids and two perioral dikinetids; 4–10 caudal cilia. Anterior and posterior main plates each with six and five windows, respectively; anterior and posterior secondary plates each with two windows, respectively.

**Voucher slides.** Two voucher slides with protargol-impregnated specimens were deposited in the Laboratory of Protozoology, Ocean University of China (registration numbers: LU-20150622-02-01, 02).

**Morphological description of Chinese population.** Size about (85–110)  $\mu\text{m}$   $\times$  (40–60)  $\mu\text{m}$  *in vivo*, barrel-shaped body with a length to width ratio of about 2: 1, usually slightly narrowed in the mid-body where the main armor plates abut. Body margin conspicuously wavy due to window domes (Figure 3A; Figure 4A). Anterior end transversely truncated and crown-like, posterior end moderately rounded to slightly pointed (Figure 3A; Figure 4A–C). Macronucleus globular to slightly ellipsoidal with a length: width ratio of about 1.2, positioned near the mid-body and close to the cell periphery. Micronucleus spherical, about 2  $\mu\text{m}$  in diameter, attached to macronucleus (Figure 3E, G; Figure 4H, I). Contractile vacuole about 10  $\mu\text{m}$  across, located near the subterminal of the body (Figure 3A; Figure 4B). Cytoplasm colorless, usually containing several food vacuoles (5–15  $\mu\text{m}$  in diameter) and numerous droplets (1–5  $\mu\text{m}$  across) (Figure 3A; Figure 4A–C).



**Figure 3** Morphology and infraciliature of the Chinese population (A–G) and South Korean population (J, K) of *Levicoleps biwae jejuensis*, *L. biwae biwae* (H, I), and *L. taehwae* (L, M)

A: Lateral view of a typical individual. B: A row of plates showing the fine structure of five tier plates. C: Ciliary pattern at apical end of the body. D, E: Lateral view of the infraciliature, arrows indicate irregular ciliary rows. F, G: Lateral view of the infraciliature, arrow indicates irregular ciliary row. H, I: Lateral view of living individual and infraciliature of *Levicoleps biwae biwae* (from Foissner et al., 2008). J, K: *Levicoleps biwae jejuensis* collected from Jeju Island (Chen et al., 2016). L, M: *Levicoleps taehwae* collected from Taehwae River, South Korea (Chen et al., 2016). Abbreviations: AO, adoral organelle; AMT, anterior main tier; AST, anterior secondary tier; CAT, caudal tier; CC, caudal cilia; CK, circumoral kinety; CO, ciliary outlet; CV, contractile vacuole; Ma, macronucleus; MB, midbar; Mi, micronucleus; PC, perioral ciliature; PMT, posterior main tier; PR, plate processes; PST, posterior secondary tier; W, plate windows. Scale bars=50  $\mu\text{m}$  (A, D–G), 20  $\mu\text{m}$  (H–M).

A armor plates *hirtus*-type and composed of six tiers: circumoral, anterior secondary, anterior main, posterior main, posterior secondary and caudal, with each tier composed of 21–24 plates

(Figure 3A, B; Figure 4A–G). Circumoral tier hardly observed *in vivo*. Anterior secondary plates about 15  $\mu\text{m}$  long, with triangle-shaped anterior end, two windows, three plate processes, and

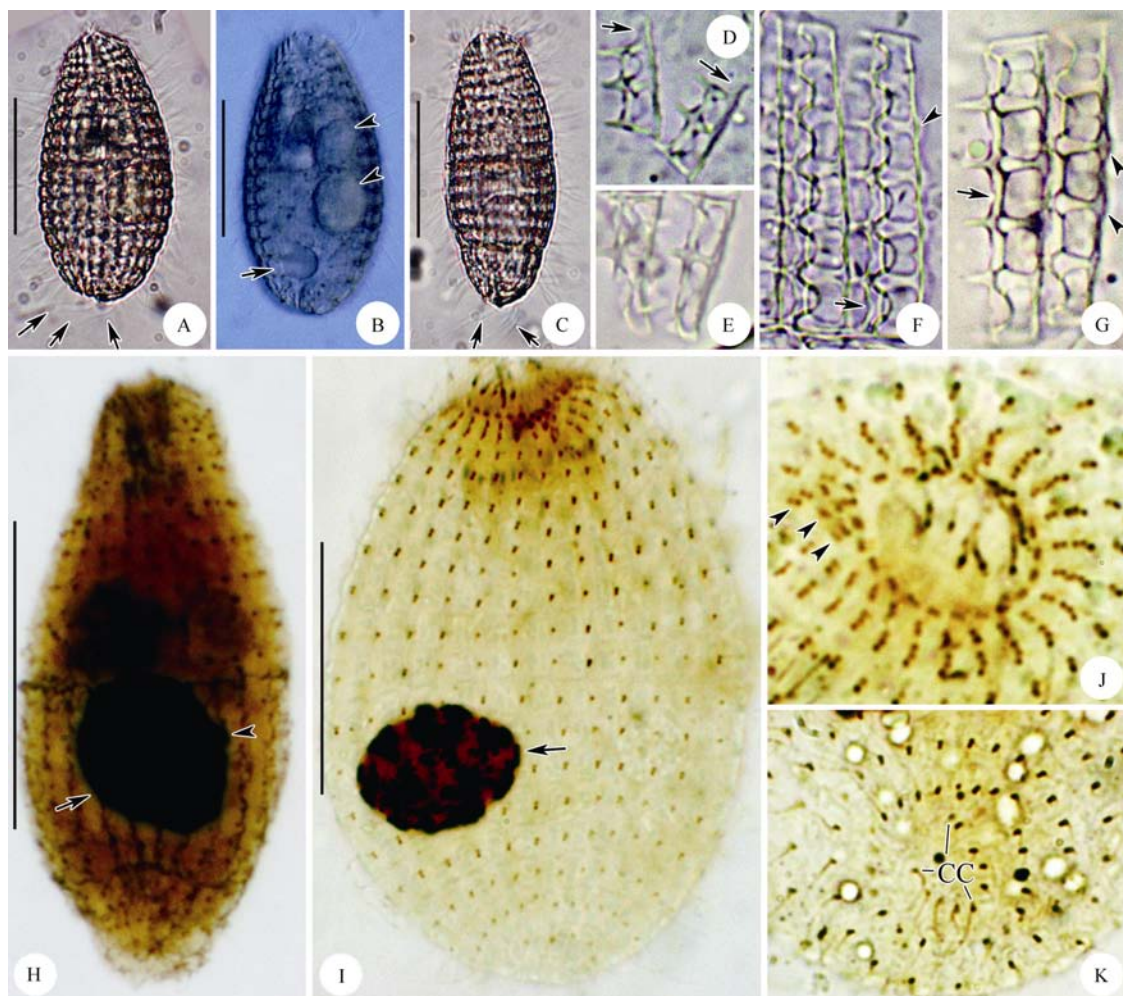


two ciliary outlets (Figure 3B; Figure 4D). Anterior main plates about 35  $\mu\text{m}$  long, with six windows gradually enlarged from the anterior to posterior, seven plate processes, and six ciliary outlets (Figure 3B; Figure 4F). Posterior main plates about 30  $\mu\text{m}$  long, with five windows, six plate processes, and five ciliary outlets (Figure 3B; Figure 4G). Posterior secondary plates trapezoidal-shaped, about 12  $\mu\text{m}$  long, with two elongated windows, three plate processes, and two ciliary outlets (Figure 3B; Figure 4E). Caudal tier about 4  $\mu\text{m}$  long with a single window (Figure 3B). The fine structure of the armor plates is shown in Figure 3B and Figure 4D-G: left margin generally smooth and slightly waved at the level of the bridge, plate processes on the right edge arranged alternately with windows and connected with inconspicuous bridges; bi-window with conspicuous radian sieve

domes. In general, 2, 6, 5, 2 windows in the middle four tiers from the anterior to posterior (Figure 3A, B; Figure 4A-G).

Oral opening occupying the central region of the anterior pole. Circumoral kinety circular and composed of dikinetids (Figure 3C; Figure 4J). Adoral organelles consisting of three parts, organelle 1 and 2 each composed of two pairs of kinetosomes, organelle 3 composed of three pairs of kinetosomes (Figure 3C; Figure 4J). Internal and external oral basket about 8  $\mu\text{m}$  and 12  $\mu\text{m}$  long, respectively, in protargol-impregnated specimens (Figure 4H).

Regularly arranged somatic cilia about 18  $\mu\text{m}$  long, forming 15 transverse circles and 21-24 longitudinal rows (Figure 3D-G; Figure 4H, I). Anterior two close dikinetids of each longitudinal ciliary row forming perioral ciliature. Six to ten caudal cilia about 25  $\mu\text{m}$  long *in vivo* (Figure 3A, D, F; Figure 4A, C, K).



**Figure 4** Microphotographs of the Chinese population of living *Levicoleps biwae jejuensis* and after protargol impregnation

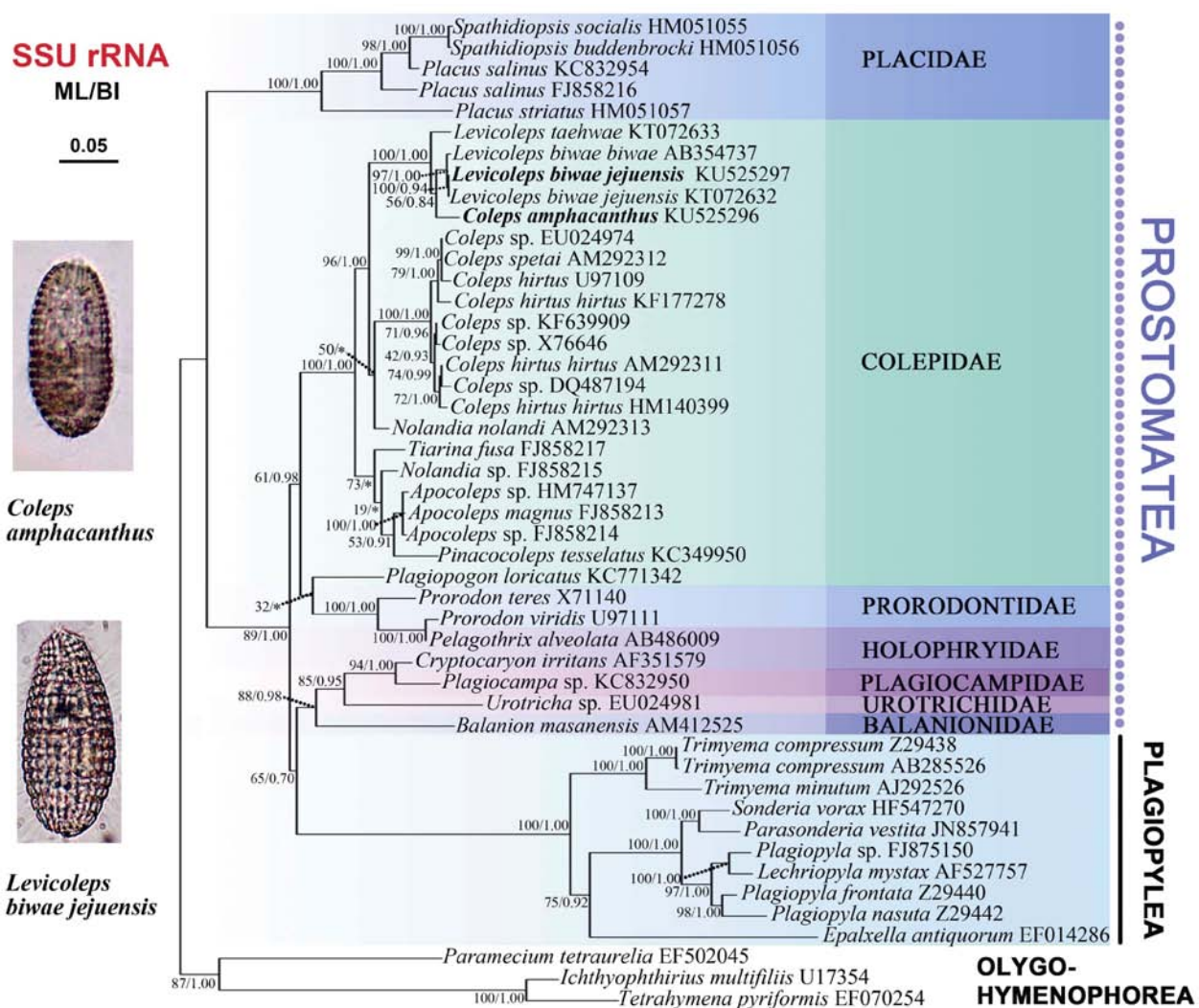
A: Lateral view of a typical individual, arrows mark caudal cilia. B: Lateral view, showing contractile vacuoles (arrow) and food vacuoles (arrowheads). C: Lateral view of a slender individual, arrows denote caudal cilia. D: Anterior secondary plates, arrows indicate pointed anterior end. E: Posterior secondary plate showing elongated windows. F, G: Anterior main plates (F) and posterior main plates (G), arrows mark ciliary outlets and arrowheads mark bulge. H: Lateral view of infraciliature, arrow marks macronucleus and arrowhead denotes micronucleus. I: Lateral view of a pressed specimen after protargol-impregnation, showing ciliary pattern, arrow marks macronucleus. J: Apical view, showing ciliary pattern at apical end, arrowheads denote three adoral organelles. K: Bottom view, showing caudal cilia. Abbreviation: CC, caudal cilia. Scale bars=50  $\mu\text{m}$ .

### SSU rRNA gene sequences and phylogenetic analyses

The SSU rRNA gene sequences of *Coleps amphacanthus* and *Levicoles biwae jejuensis* were deposited in the GenBank database with accession numbers KU525296 and KU525297, respectively. The length and GC content of the SSU rRNA gene sequence of *Coleps amphacanthus* were 1 718 bp and 44.18%, respectively, while those of *Levicoles biwae jejuensis* (Ningbo population) were 1 719 bp and 44.27%, respectively.

Forty-seven species/populations were included in the present phylogenetic analyses, containing three oligohymenophorean species as out-groups and all species from classes Prostomatea and Plagiopylea available for SSU rRNA gene sequences. In

the class Prostomatea, there were 34 species/populations representing 15 genera from seven families (Balanionidae, Colepidae, Holophryidae, Placidae, Plagiocampidae, Prorodontidae, and Urotrichidae). The topologies of the ML and BI trees were basically congruent and, therefore, a single topology was presented based on the ML tree with support values from both algorithms indicated on the branches (Figure 5). In the phylogenetic trees, Prostomatea was polyphyletic with all species grouped into three clades: Placidae formed one fully supported clade; Colepidae, Prorodontidae, and *Pelagothrix alveolata* (Holophryidae) formed the second clade (61% ML, 0.98 BI); Plagiocampidae, Urotrichidae, Balanionidae, and *Cryptocaryon irritans* (Holophryidae) formed the third clade



**Figure 5** Maximum likelihood (ML) tree inferred from SSU rRNA gene sequences showing positions of *Coleps amphacanthus* and *Levicoles biwae jejuensis*

Sequences newly obtained are in bold. Numbers near nodes represent non-parametric values of ML out of 1 000 replicates and Bayesian inference (BI) posterior probabilities. Disagreements between ML and BI are shown by asterisks. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

(88% ML, 0.98 BI), and is a sister to the class Plagiopylea (65% ML, 0.70 BI). Within the family Colepidae, both genera *Coleps* and *Levicolaps* were grouped together with high support (96% ML, 1.00 BI). All *Coleps* species were branched together, except for *Coleps amphacanthus*, which was grouped into the clade of the genus *Levicolaps*. All three *Levicolaps biwae* isolates formed a well-supported clade (97% ML, 1.00 BI), with the two isolates of *Levicolaps biwae jejuensis* grouped together.

### Hypothesis testing

Neither the genus *Coleps* nor *Levicolaps* was a monophyletic group in the present phylogenetic tree, as *Coleps amphacanthus* was always nested within the *Levicolaps* cluster. However, the constrained topology that all species of *Levicolaps* formed a monophyletic clade was not rejected at the 5% significance level ( $P=0.127$ ). The hypothesized monophyly of the genus *Coleps* was rejected ( $P=8e-008$ ), which raises the question whether the current morphological classification of *Coleps* is appropriate. Additional molecular data from taxa with indistinct spines or several caudal cilia are needed to determine whether this genus should be further split (Table 2).

**Table 2** Approximately unbiased test results

Topology constraints	Log-likelihood (-lnL)	AU value ( <i>P</i> )
Unconstrained	14143.12163	0.967
<b><i>Coleps</i></b>	<b>14157.50543</b>	<b>8e-008</b>
<i>Levicolaps</i>	14251.18767	0.127

Significant differences ( $P<0.05$ ) between the best maximum likelihood tree and constrained topologies are in bold.

## DISCUSSION

### Remarks on *Coleps amphacanthus* Ehrenberg, 1833

*Coleps* Nitzsch, 1827 is characterized by its spiny armor composed of six tiers with *hirtus*-type plates and three adoral organelles. Till now, only four species of *Coleps* have been investigated using silver staining methods: *Coleps amphacanthus* Ehrenberg, 1833, *C. elongatus* Ehrenberg, 1831, *C. hirtus hirtus* Nitzsch, 1827, *C. hirtus viridis* Ehrenberg, 1831, and *C. spetai* Foissner, 1984 (Foissner, 1984; Foissner & O'Donoghue, 1990; Foissner et al., 1994, 1999).

*Coleps amphacanthus* has been investigated several times based on living and fixed specimens (Ehrenberg, 1833; Huttenlauch, 1987; Huttenlauch & Bardele, 1987; Foissner & O'Donoghue, 1990; Kreutz & Foissner, 2006; Noland, 1925). However, standard taxonomic data were only provided by Huttenlauch (1987) and Foissner & O'Donoghue (1990). Except for smallish spines (vs. conspicuous spines in the original description and subsequent re-descriptions), the morphology of the present organism is identical with previous populations. The dissimilarity of the spines is possibly due to different environments (the definite conclusion can not be provided here, further studies on this character are necessary), and hence should be considered as a population-dependent character.

*Coleps amphacanthus* differs from most congeners by its large body size and non-single caudal cilium. Only one species,

namely *C. elongatus*, has more than one caudal cilium and should be compared with *C. amphacanthus*. The latter can be easily distinguished from the former by the number of somatic kineties (24-26 in *C. amphacanthus* vs. 14-18 in *C. elongatus*), the number of windows in the anterior and posterior main plates (8, 6 in *C. amphacanthus* vs. 5, 4 in *C. elongatus*, respectively), and the number of caudal cilia (5-10 in *C. amphacanthus* vs. 2 in *C. elongatus*).

In the phylogenetic trees, *Coleps amphacanthus* was separated from the genus *Coleps*, and clustered in the genus *Levicolaps* (Figure 5). Foissner et al. (2008) suggested that the presence or absence of armor spines is one of four principal generic features in the family Colepidae, with *Levicolaps* differing from *Coleps* mainly by its smooth armor without any spines. As previously described, *Coleps amphacanthus* has several obvious anterior and posterior spines in the original and subsequent re-descriptions (Ehrenberg, 1833; Noland, 1925; Huttenlauch, 1986; Foissner & O'Donoghue, 1990). The Ningbo population of *C. amphacanthus* has two anterior and three posterior inconspicuous smallish spines, and therefore should be assigned to *Coleps* according to the possession of spines. However, phylogenetic analyses did not support this, indicating that the presence or absence of armor spines might not be a good generic feature to separate *Coleps* and *Levicolaps*. Unfortunately, no molecular information currently exists in regards to historic populations. Additional molecular data will help reveal the correct phylogenetic position of this organism.

### Remarks on *Levicolaps biwae jejuensis* Chen et al., 2016

*Levicolaps biwae* was originally discovered in a 4-million-year-old ancient freshwater lake (Lake Biwa) in Japan (Foissner et al., 2008). Combined other biogeographic information, including the occurrence of *Planicolaps* only in Lake Tanganyika (Africa) (Dragesco & Dragesco-Kernéis, 1991), the genera *Baikalocolaps*, *Kotinia*, *Macrocoleps*, and *Tiarinella* seem to be restricted to Lake Baikal (Obolkina, 1995). Foissner et al. (2008) deduced that *Levicolaps* is a special genus from Lake Biwa. However, Chen et al. (2016) found a subspecies and other *Levicolaps* species, namely *Levicolaps biwae jejuensis* and *L. taehwae*, in South Korea. We also found a Chinese population of *L. biwae jejuensis* from a freshwater pond in Ningbo. In view of these isolations in South Korea and China, the genus *Levicolaps* does not appear to be endemic to Lake Biwa only, but is at least common to Asia.

*Levicolaps* is characterized by smooth armor composed of six tiers with *hirtus*-type plates, the absence of spines and three adoral organelles (Foissner et al., 2008). We identified our form by basic characters of armor plate, absence of spines, three adoral organelles, and several caudal cilia. Except for the number of caudal cilia and windows of the main armor plates, the organism we isolated corresponded perfectly with *L. biwae jejuensis*. At the present state of knowledge, these differences should be considered as population-dependent characters in *Levicolaps* species (Foissner et al., 2008). Moreover, this organism and *L. biwae jejuensis* (KT072632) were grouped together with strongly supported SSU rRNA trees (100% ML,



0.94 BI), and their SSU rRNA gene sequences differed by only one nucleotide. Based on morphological and molecular information, our isolate should be considered a Chinese population of *L. biwae jejuensis*. In addition, the SSU rRNA gene sequence of our isolate differed by seven nucleotides from that of *L. biwae biwae*.

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