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Morphology and Phylogeny of the Soil Ciliate *Metopus yantaiensis* n. sp. (Ciliophora, Metopida), with Identification of the Intracellular Bacteria

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ABSTRACT

The morphology and infraciliature of a new ciliate, *Metopus yantaiensis* n. sp., discovered in coastal soil of northern China, were investigated. It is distinguished from its congeners by a combination of the following features: nuclear apparatus situated in the preoral dome; 18–21 somatic ciliary rows, of which three extend onto the preoral dome (dome kineties); three to five distinctly elongated caudal cilia, and 21–29 adoral polykinetids. The 18S rRNA genes of this new species and two congeners, *Metopus contortus* and *Metopus hasei*, were sequenced and phylogenetically analyzed. The new species is more closely related to *M. hasei* and the clevelandellids than to other congeners; both the genus *Metopus* and the order Metopida are not monophyletic. In addition, the digestion-resistant bacteria in the cytoplasm of *M. yantaiensis* were identified, using a 16S rRNA gene clone library, sequencing, and fluorescence in situ hybridization. The detected intracellular bacteria are affiliated with Sphingomonadales, Rhizobiales, Rickettsiales (Alphaproteobacteria), *Pseudomonas* (Gammaproteobacteria), Rhodocyclales (Betaproteobacteria), Clostridiales (Firmicutes), and Flavobacteriales (Bacteroidetes).

MEMBERS of the ciliate genus *Metopus* Claparede and Lachmann, 1858 are widely distributed in anaerobic and microaerobic freshwater, terrestrial, and marine habitats (Bourland and Wendell 2014; Bourland et al. 2014, 2016; Corliss 1979; Da Silva-Neto et al. 2016; Jankowski 1964a, b; Lynn 2008). Despite about 81 species and 25 sub-species of this genus having been recorded (Roskov et al. 2016), only a few *Metopus* species have been investigated, using modern morphological methods and sequencing for phylogenetic inference. Morphologically, this genus can be easily recognized because of the anteriorly twisted body and the oblique or spiraling adoral zone of polykinetids, which is overhung by the preoral dome (Foissner et al. 1992; Jankowski 1964a,b; Kahl 1927; Vďačný and Foissner 2017).

Cell biology and ecology of *Metopus* species are also characteristic among ciliates. They have symbiotic prokaryotes and hydrogen-forming organelles (called hydrogenosomes) instead of classical mitochondria as an adaptation to the anaerobic environment (Hackstein et al. 1999; Müller 1988). The anaerobic habitat and the special organelles have motivated many scientists to study the endosymbionts of *Metopus*, of which most focused on the methanogenic archaea (van Bruggen et al. 1984, 1986; Embley and Finlay 1993; Embley et al. 1992a,b; Fenchel and Finlay 1991; Müller 1993; Narayanan et al. 2009). However, still little is known about the bacteria associated with these organisms.

In this study, we isolated and investigated the morphology and ciliary pattern of a new soil metopid species from China. We sequenced the 18S rRNA gene of this new species and two known *Metopus* species. Furthermore, the intracellular bacteria of the new ciliate species, also called digestion-resistant bacteria (DRB; Gong et al. 2016), were identified, using molecular methodologies.
MATERIALS AND METHODS

Collection and ciliate cultivation

*Metopus yantaiensis* n. sp. was discovered in a coastal soil sample originally from an apple orchard near Yantai City, Shandong, China (37°57′73″N, 120°66′51″E). The upper layer of the soil with a small amount of litter was collected, air-dried for two weeks, and stored in a plastic bag. Later, the sample was investigated, using the nonflooded Petri dish method as described by Foissner et al. (2002), with some dried and sterilized pieces of grass being added and mixed to increase the organic matter and filter capacity of the very fine-grained soil. From the nonflooded Petri dish culture (NFPC), a few ciliate cells were picked up with a micropipette, washed and incubated into mineral water supplied with sterilized chopped grass to establish the raw cultures (RC). Some cells were picked from the RC, using a fine pipette, and transferred onto a microscope slide with a few drops of filtered water; resting cysts were then formed after maintaining for a few days in a wet chamber. Two known species, *Metopus contortus* (Quennerstedt, 1867) Kahl, 1932 and *Metopus hasei* Sondheim, 1929 were sampled from marine anaerobic sediments of the Swan Lake lagoon, Rongcheng, and moss from the Kunyu Mountain, Yantai, Shandong, China, respectively.

Morphological observations

Living cells were studied, using bright field and differential interference contrast microscopy at magnifications of 100–1,000×. The ciliary and nuclear patterns were revealed, using the protargol impregnation method of Wibert (1975). Counts and measurements of prepared specimens were performed at a magnification of 1,000×. Drawings of live cells were based on free-hand sketches, while those of impregnated specimens were made, using a drawing device.

DNA extraction, PCR amplification, clone libraries, and sequencing

To extract genomic DNA of the protists and associated bacteria, ciliate cells from the raw cultures were washed three times with 0.22 μm-filtered mineral water, and then starved for about 18 h to allow digesting bacterial preys. The cells were washed again to minimize contamination. The individuals were transferred into 0.2 ml PCR tubes with a small drop of water (0.5 μl) for DNA extraction, and up to 20 cells were mounted on slides for subsequent FISH assays.

Genomic DNA was extracted with REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO) according to Gong et al. (2014). The 18S rRNA gene was PCR amplified with the primers 82F (5′-GAAACTCGAATGCTC-3′) and Euk B (5′-TGATCCCTTCTGAGGTTCACCTAC-3′) or 1520R (5′-CYGGAGGTTCACCTAC-3′). The PCR reactions were performed in a thermal cycler (Biometra, Göttingen, Germany) with the following program: prerun of 5 min at 94 °C; 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 2 min; and then a final extension step at 72 °C for 10 min. To inspect the ingested but not digested bacteria and archaea within these ciliates, we amplified the bacterial 16S rDNA, using the primers 27F (5′-AGACTTGTGATCMTGGCTCAG-3′) and 1492R (5′-ACGGTACCTGGTATCAGAT-3′), and the following program: prerun of 5 min at 94 °C; 30 cycles of denaturation at 94 °C for 90 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min; and then a final extension step at 72 °C for 10 min. The archaeal 16S rDNA was amplified with the Archaea-specific primer 21F (5′-TCCGGTTGATCCYGCCGG-3′) and the universal primer 1492R. The amplified PCR products were purified, using the TiANGen Midi Purification Kit (Tiangen, Shanghai, China), and cloned into a PTZ57R/T vector, using InstaClone PCR Cloning Kit (Thermo Scientific, Shanghai, China), and then transferred into Escherichia coli DH5α cells. Multiple positive clones with ciliate 18S (20) and bacterial 16S rRNA (46) gene inserts were selected randomly and screened, using restricted fragment length polymorphism (RFLP) analysis with the restriction enzyme Haelll under the conditions recommended by the supplier (Fast digest, Thermo Scientific). Sequencing in both directions was carried out on an ABI 377 automated sequencer (Sangon, Shanghai, China).

Phylogenetic analysis

The new 18S rRNA gene sequences of *M. yantaiensis* n. sp., *M. contortus*, and *M. hasei*, along with metopid, clevelandellid, caenomorphid, litostomean and spirotrich sequences retrieved from the GenBank database, were aligned, using MAFFT v.7 (Katoh and Standley 2013). Three karyorelicteans were used as the outgroup species. The alignment was refined manually to excise hypervariable regions, using BioEdit 7.0 (Hall 1999), resulting in 21 taxa and 1,643 nucleotide positions. For the newly obtained bacterial 16S rRNA gene sequences, we removed chimeric sequences identified by Bellerophon (Huber et al. 2004). Closely related bacterial sequences were retrieved from the GenBank and aligned with these newly obtained. The final alignment for the bacteria comprised 43 taxa and 1,423 nucleotide positions.

The maximum likelihood (ML) trees of the 18S and 16S rRNA genes were constructed, using RAxML-HPC v7.2.5 (Stamatakis et al. 2008). Nodal supports in ML analyses came from 1,000 bootstrap replicates, using heuristic searches. Both analyses employed the GTR+ G + I + I model, selected according to the Akaike information criterion in jModelTest 2.1 (Darriba et al. 2012). The Bayesian inference (BI) analysis was performed, using MrBayes v.3.2.1 (Ronquist and Huelsenbeck 2003) for both the 16S and 18S rRNA genes. Markov chain Monte Carlo (MCMC) simulations were run, with two sets of four chains, using the default settings, for 3,000,000 generations, sampling every 1,000 generations. The first 25% of sampled trees were considered burn-in and discarded, and the remaining trees were used to
generate a consensus tree and to calculate the posterior probabilities.

To test competing evolutionary scenarios, the best constrained trees were created and compared with the unconstrained ML tree. A per-site log-likelihood file for the resulting constrained topologies and the unconstrained ML topology were generated in RAxML-HPC v7.2.5 and then subjected to the approximately unbiased (AU) test (Shimodaira 2002) as implemented in CONSEL (Shimodaira and Hasegawa 2001).

**Fluorescence in situ hybridization (FISH)**

Whole-cell hybridization was according to (Gong et al. 2016). Briefly, starved ciliate cells fixed with Bouin’s solution (50%, final concentration) were dropped onto microscopic slides (SuperFrost Plus) and air dried at room temperature. Before FISH assay, the slides were washed in distilled water and then progressively dehydrated via an ethanol gradient (30%, 50%, 80%, and 100%). The specimens on the slides were incubated at 46 °C for 3 h in hybridization buffer (20 mM Tris–HCl at pH 8.0), 0.9 M NaCl, 0.01% SDS = sodium dodecyl sulphate, and 30% formamide), the Cy3-labeled eubacterial probes (EUB338, EUB338I, and EUB338II) and the Alexa Flour 448-labeled archaean probe (Arch915) with a final concentration of 5 ng/μl. After hybridization, the slides were washed for 15 min at 48 °C with washing buffer, and then rinsed with chilled double-distilled water. The protist cells were mounted with antifade mounting medium (Beyotime, China) mixed with DAPI (50 ng/ml) and observed under an fluorescence microscope (Olympus BX51, Tokyo, Japan) with green- and blue-light excitation for Cy3 and Alexa Flour 448, respectively, and UV excitation for DAPI signals. Photomicrographs were captured with a digital camera (SPOT Imaging Solutions, Sterling Heights, MI).

**Sequence accession numbers**

The ciliate 18S and bacterial 16S rRNA gene sequences newly obtained in this study have been deposited in the GenBank database under the accession numbers KY432957–KY432959 and KY432945–KY432956, respectively.

**Terminology**

Terminology is according to Foissner and Agatha (1999) and Vd’ačný and Foissner (2017). Classification is according to Jankowski (2007).

**RESULTS**

**Morphological description of Metopus yantaiensis n. sp.**

The size in vivo is 65–90 × 30–50 μm, usually about 75 × 40 μm in the nonflooded Petri dish cultures (NFPC), while it is 75–105 × 35–55 μm (about 90 × 45 μm on average) in the raw cultures (RC) (Fig. 1–3 and Table 1). The body is ellipsoidal with a length:width ratio of 2:1 on average; the preoral portion is about 40–60% (mean 45%) of the body length (Table 1); the body is distinctly twisted anteriorly due to the sigmoidal preoral dome (Fig. 1A, B, D, G, I, 2A–G, 3A–E). The oral area belongs to the Type 1 pattern (Vd’ačný and Foissner 2017), i.e. the preoral dome is flat, distinctly projects from the body proper, extends perpendicularly or inclined about 45° (in some protargol-impregnated specimens from RC, Fig. 3A) to the main body axis, and merges into mid-body at the right half of dorsal side. The dome lip is prominent and the side stripe forms a deep channel completely overhanging the adoral zone of polykinetids (Fig. 1A, B, G, 2A). The central dome portion is convex, the dome brim is distinctly twisted, thus it is broadly sigmoidal in the top view and forms a sharp angle with the dorsal side anteriorly (arrows in Fig. 1F, 2D, 3G). The postoral body portion is ellipsoidal, the ventral and left sides are distinctly convex, while the right and the dorsal sides are flat to slightly convex. The posterior third of the body has distinct longitudinal cortical folds during the systole of the contractile vacuole, thus the shape of the posterior end is rather variable ranging from narrowly to broadly rounded; cells from the NFPC are invariably broadly rounded posteriorly (Fig. 2F, G, 3C). The macronucleus is in the preoral dome, reniform, and contains numerous nucleoli in the protargol-impregnated specimens. The micronucleus is globular to ellipsoidal, usually attached to the ventral anterior end of the macronucleus (Fig. 1A, D, F, I, 2A, D, L, 3A–G). The cytopyge is slit-like and subterminal on ventral side (Fig. 1B, 2I, 3C). The contractile vacuole is large (up to 25 μm in diameter) and located terminally; likely, it opens into the cytopyge because an excretory pore was not found (Fig. 1A, D, 2A, C, E). The cortex is hyaline and furrowed along the ciliary rows, contains scattered, colorless cortical granules (Fig. 1A, C, 2C, D, I–K, 3E). Underneath the cortex, there are numerous, colorless, globular hydrogenosomes 1–1.5 μm across (Fig. 1C, 2J, K, 3E), whose size and location are similar to those described in M. contortus (Biagini et al. 1997; Esteban et al. 1995) and M. hasei (present study; see Fig. 4F). The cytoplasm is colorless, hyaline, containing many, 2–3-μm sized lipid droplets mainly in the anterior cell half; the preoral dome is packed with cytoplasmic granules 0.5–1 μm across, these granules do not stain with protargol and were found in all observed specimens from the NFPC and RC with variable intensities (Fig. 1A, 2C, F, L). Well-fed specimens contain numerous food vacuoles 5–18 μm across, with purple sulfur bacteria and bacterial spores (Fig. 1A, 2A, B, E, F, 3A, 7A). The cells swim moderately rapidly and continuously by rotating about the main body axis.

In vivo, the ordinary somatic cilia are 12–15 μm long, whereas the perizonal cilia are slightly longer (about 18–20 μm); the three to five elongated (25–30 μm long) caudal cilia are straight and immotile in vivo, but often not recognizable in protargol-impregnated specimens (Fig. 1A, 2A, B, 3E). There are about 19 ciliary rows (including the dome kinetics), composed of widely spaced, longitudinally
oriented (parallel to kinety axis) dikinetids having associated a cilium with the posterior basal body, except for the diciliated dikinetids in the anterior portion of the dome kineties. The ventral (postoral) kineties commence underneath the adoral zone of polykinetids (AZP) and are slightly shortened posteriorly, forming a blank circular area containing the cytopyge (asterisk in Fig. 1B). Underneath the proximal end of the adoral zone of polykinetids and the buccal cavity, a few scattered dikinetids occur (Fig. 1B). The dorsal kineties are anteriorly shortened from right to left and slightly elongated posteriorly across the pole, terminating at the blank area containing the cytopyge. Invariably three widely spaced, bipolar dome kineties, extend in a spiraled course leftwards onto the preoral dome and converge near the distal end of the perizonal stripe (PS). Dome kinety 1 is separated from the perizonal stripe kinety 5 (PS5) by a wide space; the dikinetids in the posterior portion are irregularly oriented (Fig. 1D, I, 2D, 3B, D). The PS is on the brim of the preoral dome and is slightly longer than the adoral zone of polykinetids (occupies about 61% of body length on average, Table 1). It is composed of five narrowly spaced kineties (PS1–PS5).

Figure 1. Metopus yantaiensis n. sp. from life (A, C, G, H) and after protargol impregnation (B, D–F, I). (A) Ventral view of a representative specimen showing the body shape, the preoral dome containing the nuclear apparatus and the cytoplasmic granules, the slight furrows along the somatic kineties and the elongated caudal cilia. Note the convex left side and the concave right side of the postoral region. (B, E) Ventral view and details of the perizonal stripe of the holotype specimen showing the oral ciliature, the loosely arranged dikinetids underneath the buccal cavity (arrows), the blank area containing the cytopyge (asterisk), and the pharyngeal fibers (arrowhead). The perizonal stripe kineties 1–3 form “false kineties” and alternately arranged with perizonal kineties 4 and 5. (C) Surface view showing the loosely arranged cortical granules and the hydrogenosomes. (D) Dorsal view showing the nuclear apparatus and the bipolar dome kineties. Note the wide area between the perizonal stripe kinety 5 and the dome kinety 1. (F) Anterior polar view. The arrow indicates the sharp corner made by the wide preoral dome and the dorsal side. (G, H) Ventral views showing the variability of the body shape. (I) Right side view showing the wide area between the dome kinety 1 and the perizonal kinety 5, the wide space underneath the proximal end of the perizonal stripe (asterisk) and the irregularly arranged dikinetids in the posterior portion of dome kinety 1 (arrowheads). AZP = adoral zone of polykinetids; CC = caudal cilia; CG = cortical granules; CV = contractile vacuole; DK1–3 = dome kineties; FK = “false” kineties; H = hydrogenosomes; MA = macronucleus; MI = micronucleus; PM = paroral membrane; PS(1–5) = perizonal stripe kineties; SS = side stripe. Scale bars = 20 μm.
Figure 2. *Metopus yantaiensis* n. sp. from life. (A–C) Ventral views of different specimens, showing the furrows along the ciliary rows (arrowheads), the elongated, straight caudal cilia, the terminal contractile vacuole (asterisks), the food vacuoles, which are full of bacteria and bacterial spores. The nuclear apparatus is invariably in the preoral dome. (D) Right side view showing the dome kineties, the proximal end of the perizonal ciliary stripe (arrowhead), and the sharp corner made by the distal end of the preoral dome and the dorsal side (arrow). (E) Ventral view of a well-fed specimen showing the numerous food vacuoles, the large, terminal contracticle vacuole (asterisk) and the fecal mass containing undigested bacterial spores (arrowheads). (F) Ventral view of a well-fed specimen with broadly rounded posterior end. (G) Right side view of starved specimen after the systole of the contractile vacuole with very narrow posterior end (arrowheads). (H) The resting cyst is covered by about 1 μm thick wall (arrowheads). (I) Ventral view of the postoral body portion showing the furrows along the ciliary rows and the cytopyge. (J, K) The cortical granules are inconspicuous (black arrowheads). The hydrogenosomes are globular and numerous (white arrowheads). (L) Right side view showing the dense cytoplasmic granules in the preoral dome (arrowheads), the paroral membrane and the pharyngeal fibers. AZP, adoral zone of polykinetids; CC = caudal cilia; CV = contractile vacuole; CY = cytopyge; DK1–3 = dome kineties; F = furrows; FV = food vacuoles; MA = macronucleus; MI = micronucleus; PM = paroral membrane; PS = perizonal ciliary stripe; SS = side stripe. Scale bars = 30 μm (in A–G), 15 μm (in H, I, L) and 10 μm (in J, K).
made of widely spaced, ciliated dikinetids, with the PS4 and PS5 slightly shortened anteriorly and posteriorly. The dikinetids of the perizonal rows 1–3 (PS1–3) form about 50 “false kineties” arranged alternately to the dikinetids of the PS4 and PS5. Posterior to the proximal end of the PS, a wide triangular space occurs (Fig. 1A–E, I, 2A–G, 3A–G).
The adoral zone of polykinetids is sigmoidal, commencing at the anterior left margin of the preoral dome and extending obliquely to the right margin of the ventral side, where it plunges into the buccal cavity. It occupies about 45% of body length on average and comprises about 26 polykinetids. There are two types of polykinetids differing distinctly in the structure: the distal polykinetids are cuneiform, i.e. they are composed of two long rows and one short row of basal bodies at their anterior right ends; whereas the proximal polykinetids are rectangular and composed of about three rows of basal bodies (Table 1 and Fig. 1A, B, F–H, 2A, E, 3A, C, E–G). The paroral membrane is situated near the proximal end of the adoral zone of polykinetids, extends to the undersurface of the preoral dome, and comprises a single line of ciliated basal bodies (Fig. 1A, B, 2E, L, 3A, C–F). The cytopharyngeal fibers form a long, dorsally directed funnel (Fig. 1A, B, 2L, 3B, D). Dividers and conjugants were not observed.

**Table 1.** Morphometric data on *Metopus yantaiensis* n. sp.

| Characteristics                       | Material   | Mean  | SD   | SE  | CV   | Min  | Max   | n  |
|--------------------------------------|------------|-------|------|-----|------|------|-------|----|
| Body, length                         | RC         | 78.3  | 78.0 | 6.4 | 1.4  | 8.2  | 65.0  | 95.0| 21 |
|                                       | NFPC       | 61.7  | 60.0 | 5.0 | 1.1  | 8.1  | 55.0  | 74.0| 21 |
| Body, total width b                   | RC         | 37.2  | 40.0 | 4.2 | 0.9  | 11.1 | 30.0  | 55.0| 21 |
|                                       | NFPC       | 34.1  | 35.0 | 5.2 | 1.1  | 15.2 | 26.0  | 42.0| 21 |
| Body, width at cytostome              | RC         | 30.3  | 30.0 | 3.9 | 0.9  | 12.9 | 22.0  | 36.0| 21 |
|                                       | NFPC       | 30.0  | 30.0 | 3.5 | 0.8  | 11.7 | 22.0  | 35.0| 21 |
| Body, maximum postoral width          | RC         | 27.7  | 28.0 | 3.7 | 0.8  | 13.5 | 21.0  | 34.0| 21 |
|                                       | NFPC       | 30.3  | 29.0 | 4.2 | 0.9  | 13.9 | 25.0  | 40.0| 21 |
| Body length:total width, ratio b      | RC         | 2.1   | 2.0  | 0.3 | 0.1  | 11.6 | 1.8   | 2.6 | 21 |
|                                       | NFPC       | 1.9   | 1.9  | 0.3 | 0.1  | 18.4 | 1.4   | 2.5 | 21 |
| Body, length in vivo                  | RC         | 87.3  | 90.0 | 8.6 | 2.0  | 9.8  | 73.0  | 104.0| 21 |
| Body, total width in vivo b           | RC         | 43.8  | 40.0 | 5.7 | 1.3  | 12.9 | 35.0  | 55.0| 19 |
| Body length:total width, ratio in vivo b | RC       | 2.0   | 2.0  | 0.3 | 0.1  | 14.6 | 1.5   | 2.6 | 19 |
| Anterior cell end to proximal end of AZP, distance | RC | 35.2  | 35.0 | 4.5 | 1.0  | 12.9 | 30.0  | 47.0| 21 |
| Distance anterior cell end to proximal end of AZP: body length, ratio in % | RC | 45.0  | 45.0 | 5.0 | 1.1  | 11.2 | 37.5  | 57.5| 21 |
| Anterior cell end to proximal end of PS, distance | RC | 47.6  | 48.0 | 4.4 | 1.0  | 9.2  | 40.0  | 56.0| 21 |
| Distance anterior cell end to proximal end of PS: body length, ratio in % | RC | 61.0  | 61.4 | 6.3 | 1.4  | 10.3 | 50.0  | 75.3| 21 |
| Macronucleus, length                   | RC         | 26.1  | 26.0 | 1.7 | 0.4  | 6.6  | 23.0  | 29.0| 21 |
| Macronucleus, width                    | RC         | 10.2  | 10.0 | 1.4 | 0.3  | 13.4 | 9.0   | 14.0| 21 |
| Anterior cell end to posterior         | RC         | 26.9  | 27.0 | 4.5 | 1.0  | 16.9 | 19.0  | 35.0| 21 |
| end of macronucleus, distance         | NFPC       | 22.8  | 24.0 | 5.3 | 1.2  | 23.1 | 15.0  | 35.0| 21 |
| Distance anterior cell end to posterior | RC      | 33.8  | 34.3 | 5.1 | 1.1  | 15.0 | 23.8  | 47.9| 21 |
| end of macronucleus: body length, ratio in % | NFPC | 36.8  | 38.5 | 7.1 | 1.2  | 19.3 | 26.7  | 52.6| 21 |
| Micronucleus, length                   | RC         | 5.2   | 5.0  | 0.6 | 0.1  | 11.6 | 4.0   | 7.0 | 21 |
| Micronucleus, width                    | RC         | 5.0   | 5.0  | 0.7 | 0.2  | 14.0 | 4.0   | 7.0 | 21 |
| Macronucleus, number                   | RC         | 1.0   | 1.0  | 0.0 | 0.0  | 0.0  | 1.0   | 1.0 | 21 |
| Macronucleus, number                   | RC         | 1.0   | 1.0  | 0.0 | 0.0  | 0.0  | 1.0   | 1.0 | 21 |
| Somatic ciliary rows, number           | RC         | 19.3  | 19.0 | 1.0 | 0.2  | 5.0  | 18.0  | 21.0| 21 |
| Caudal cilia, number in vivo          | RC         | 3.6   | 3.0  | 0.9 | 0.2  | 25.3 | 3.0   | 5.0 | 15 |
| Adoral membranelles, number           | RC         | 26.4  | 27.0 | 1.7 | 0.4  | 6.4  | 21.0  | 29.0| 23 |
| Paroral membrane, length              | RC         | 15.4  | 15.0 | 1.2 | 0.3  | 7.6  | 13.0  | 18.0| 21 |
| Perizonal ciliary rows, number        | RC         | 5.0   | 5.0  | 0.0 | 0.0  | 0.0  | 5.0   | 5.0 | 23 |
| Plase kineties, number                | RC         | 50.8  | 50.0 | 4.5 | 1.0  | 8.8  | 40.0  | 60.0| 21 |

AZP = adoral zone of polykinetids; CV = coefficient of variation in %; Mean = arithmetic mean; M = median; Max = maximum; Min = minimum; n = number of specimens investigated; PS = perizonal ciliary stripe; SD = standard deviation; SE = standard error of mean.

aData based, if not mentioned otherwise, on protargol-impregnated, randomly selected specimens from nonflooded Petri dish culture (NFPC) and raw cultures (RC). Measurements in µm.

bWidth including margins of preoral dome.

cNumber includes dome kineties.

dSee description.

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Metopus yantaiensis and Associated Bacteria

Omar et al.

Resting cysts

The seven-day-old resting cysts of *M. yantaiensis* n. sp. are colorless, spherical, about 45 µm in diameter on
average. The cyst wall is about 1 μm thick, smooth, and structureless. The cytoplasm contains lipid droplets and a globular macronucleus (Fig. 2H).

Occurrence and ecology
As yet only found at the type locality (see above). The species appeared in the old culture after formation of anaerobic microsites and could be cultivated in raw cultures with mineral water to which some dried and sterilized pieces of grass were added. It grew well together with Colpoda maupasi in the cultures that were supplied with much detritus. The cell density of the new species can be quite high in the early stages of cultivation and declined later when flagellates became dominant. It cannot be excluded that M. yantaiensis n. sp. is aerotolerant since it is able to survive for a few days in open cultures.

Notes on the Shandong populations of Metopus contortus and Metopus hasei
Since the morphology and infraciliature correspond well with those previously described populations of M. contortus and M. hasei, only some key features of our populations are described here (Fig. 4).

**Description of the Shandong population of Metopus contortus**
The size is 100–140 × 30–40 μm in vivo. The body shape is ellipsoidal; the preoral dome is flattened. The elongate ellipsoidal macronucleus is in the anterior half of body, it is about 40 × 15 μm in size; the micronucleus is globular and about 5 μm across. There are 30–40 narrowly spaced kineties; five perizonal rows extending almost to mid-body; rows 1–3 form “false kinetics”; four caudal cilia up to 25 μm long. The adoral zone consists of 35–40 polykinetids; the paroral membrane is about 25 μm long, comprises a single file of ciliated basal bodies (Fig. 4A–C). We identify this species based on the number of somatic kineties, the shape of the preoral dome, the length of the perizonal stripe, and the elongated caudal cilia (Dragesco 1996; Esteban et al. 1995; Foissner et al. 2002; Kahl 1932).

**Description of the Shandong population of Metopus hasei**
The size in vivo is 80–110 × 15–30 μm, the length:width ratio is 3–8:1, usually about 4:6:1. The preoral dome is slightly sigmoidal and the dome brim is inconspicuous. The postoral cell portion is cylindroidal with a rounded posterior end. The nuclear apparatus is in the anterior cell half...
and left of the adoral zone of polykinetids. The contractile vacuole is terminal. The four or five caudal cilia are up to 40 μm long in length. About 13–15 comparatively widely spaced ciliary rows. The perizonal ciliary stripe occupies about 25% of body length and is slightly shorter than the adoral zone of polykinetids (Fig. 4D–F). Our population matches the type (Sondheim 1929), the Austrian (Foissner 1981), the Namibian, and the South African populations (Foissner and Agatha 1999).

**Phylogenetic analyses**

The sequenced 18S rRNA gene fragment of *M. yantaiensis* n. sp. is 1,588 bp long and has a GC content of 44.33%. The sequence of the Shandong population of *M. contortus* exhibited 98% similarity with the Danish population (Hirt et al. 1995). The topologies of the ML and BI trees were almost identical (Fig. 5). *Metopus yantaiensis* n. sp. closely clusters with *M. hasei* in the trees but without support from ML (< 50) and BI (0.62). The clade of *yantaiensis-hasei* appears to be a sister of the two *Atopospira* species and *Parametopidium circumlabens* with weak support from BI (0.93). Interestingly, the three *Metopus* species (*M. yantaiensis, M. hasei*, and *M. laminarius*) form together with *Atopospira* spp., *Parametopidium* (family Metopidae), *Nyctotheroides* spp., *Nyctotherus ovalis* (family Nyctotheridae), and *Clevelandella* spp. (family Clevelandellidae) a highly supported clade (ML 98, BI 1.00). The other five *Metopus* species (i.e. *M. contortus, M. striatus, M. fuscus, M. setosus*, and *M. es*) form a sister group to this clade and are scattered among the genera *Palmarella* and *Brachonella*. *Heterometopus palaearcium* (Kahl, 1927) diverges at the base of the *Metopus + Clevelandella* clade with full bootstrap support.

**Figure 5** A maximum likelihood (ML) tree based on 18S rRNA genes, showing the phylogenetic positions of *Metopus yantaiensis* n. sp. and the Chinese populations of *Metopus contortus* and *Metopus hasei*. Newly obtained sequences are highlighted in bold. Arrows indicate clades of the class Armophorea. GenBank accession numbers follow species names. The best-fit GTR + G + I model was used. Bootstrap values lower than 50% and posterior probabilities in Bayesian inference (BI) lower than 0.5 were not shown.

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support in the ML and BI analyses. The AU tests of the null hypotheses concerning the monophyly of the genus *Metopus* and the order Metopida result in p-values of 3e-004 and 0.047, respectively, indicating that neither the genus nor the order are monophyletic (Table 2).

**Identification of digestion-resistant bacteria in *Metopus yantaiensis* n. sp.**

Of the 46 bacterial clones of 16S rRNA genes, 12 sequences belonging to seven RFLP types were obtained (Fig. 6, 7). The phylogenetic analysis show that the digestion-resistant bacteria (DRB) belong to three bacterial phyla: the Bacteroidetes, Firmicutes, and Proteobacteria. Three proteobacterial classes were detected: the Alphaproteobacteria (*relative abundance 85%*), Betaproteobacteria (*2.5%*), and Gammaproteobacteria (*7.5%*). Most (79%) of the alphaproteobacterial DRB were identified to be *Sphingomonas aquatilis* (100% similarity) within the family Sphingomonadaceae; the remaining alphaproteobacteria were probably *Rhodopseudomonas palustris* (99%) of the family Bradyrhizobiaceae. The phylotype of the family Rickettsiaceae was minor (2.5%) in the DRB assemblage, sharing a sequence similarity of 89% with *Rickettsia prowazekii.* The Betaproteobacteria were represented only by *Azospira oryzae* (99% similarity). The Gammaproteobacteria were represented by the family Pseudomonadaceae, most likely the species *Pseudomonas alcaligenes* (99% similarity). Two phylotypes, sharing 96% and 99% sequence identities with *Clostridium* (Firmicutes, Clostridiales) and *Cloacibacterium normanense* (Bacteroidetes, Flavobacteriales), respectively, were minor, each accounting for 2.5% in the DRB assemblage. Attempts to amplify the archaeal DNA failed.

**Fluorescence in situ hybridization (FISH)**

The large quantities of bacteria inside the food vacuoles of nonstarved specimens (Fig. 2A–C, F, 3A, 7A, E) indicate that *M. yantaiensis* n. sp. is a significant bacterivore. However, the FISH assays showed that the numbers of bacteria inside the cytoplasm were reduced in the starved specimens, while a large number of these ingested bacteria remained and were viable for a long time (at least 18 h) (Fig. 7B–D), suggesting the digestion resistance nature of these bacteria. No archaeal cells were detected in the cytoplasm of *M. yantaiensis.*

**DISCUSSION**

**Comparison of *Metopus yantaiensis* n. sp. with similar species**

*Metopus yantaiensis* n. sp. is most similar to *M. inversus* (Jankowski 1964) Foissner and Agatha, 1999 in body outline, shape and location of the nuclear apparatus (reniform macronucleus in the preoral dome), and length of the perizonal ciliary stripe (61% of body length). However, they differ in the following features: (i) the number of somatic kineties (18–21 vs. 22–25), (ii) the number of the adoral polykinetids (21–29 vs. 29–37), (iii) the shape of the dome brim (thick vs. thin and hyaline), (iv) the presence vs. absence of a cytoplasmic granule accumulation in the preoral dome, (v) the number of “false kineties” (40–60 vs. 58–74), (vi) the length of the dome kinety 1 (bipolar vs. as long as the perizonal stripe); and (vii) the presence vs. absence of caudal cilia. Actually, the presence/absence of caudal cilia has been considered an important feature for distinguishing *Metopus* species by previous investigators (e.g. Esteban et al. 1995; Foissner and Agatha 1999; Foissner et al. 2002; Kahl 1927).

The cylindroidal specimens of *M. yantaiensis* n. sp. from the raw culture resemble *M. hasei* (Fig. 4D–F), as described by Foissner and Agatha (1999) and the present study, in body size and the presence of caudal cilia. However, both can be distinguished by the maximum length:width ratio (up to 2.6:1 vs. 8:1 in the Chinese population, 3.6 in the Namibian population and 7.6:1 in the South African population of *M. hasei*), the location of the nuclear apparatus (in vs. posterior to the preoral dome), the length of the perizonal stripe (61% vs. 35% of body length), and the number of somatic ciliary rows (18–21 vs. 10–16).

The protargol-impregnated specimens of *Metopus yantaiensis* n. sp. from the nonflooded Petri dish culture are stout (Fig. 3C) and thus they look similar to *Metopus gibbus* Kahl, 1927; as described by Foissner et al. (2002). However, these two species can be easily distinguished by their body sizes (65–90 × 30–50 μm vs. 30–55 × 18–35 μm), the numbers of somatic kineties (18–21 vs. 12–15) and adoral polykinetids (21–29 vs. 13–19), the distributional pattern of cortical granules (scattered vs. dense), and the accumulation of cytoplasmic granules in the preoral dome (present vs. absent); the latter is an important feature for delimiting *Metopus* species (Bourke and Wendell 2014; Bourland et al. 2014; Foissner et al. 2002; Kahl 1927). Moreover, the caudal cilia in *M. yantaiensis* n. sp. do not form synclia as in *M. gibbus*.
Phylogeny of *Metopus* species

With the newly obtained 18S rRNA gene sequences for *M. yantaiensis* and *M. hasei*, our analysis clearly shows that the class Armophorea is monophyletic (p-value = 0.329), while the genus *Metopus* is nonmonophyletic, which is consistent with Bourland et al. (2014, 2016) and da Silva-Neto et al. (2016). *Metopus yantaiensis* is more closely related to *M. hasei*, *Atopospira* spp., and *Parametopidium circumlabens* than to other congeners in the phylogenetic trees, for which the diversification of morphological characters is not well understood (Table S1). The clade comprising these six metopid species (including *M. laminarius* which is highly similar to *M. hasei*, except for the absence of the caudal cilia) and the clevelandellid taxa is strongly supported (ML 98, BI 1.00), suggesting that these metopids and clevelandellids are phylogenetically related as shown in previous studies (Affa’a et al. 2004; Bourland et al. 2014; van Hoek et al. 1998, 1999; Li et al. 2016; Lynn and Wright 2013; da Paiva et al. 2013; da Silva-Neto et al. 2016). Nevertheless, this close relationship cannot be supported by morphological synapomorphies. Clearly, more detailed morphological and molecular studies are necessary for understanding the diversification of the armophorids and the origin of clevelandellids.

Digestion-resistant bacteria

Recent data have shown that some bacterial preys can be ingested but not digested by some marine and freshwater ciliates (Gong et al. 2014, 2016). However, information on bacteria that can evade digestion by protists living in terrestrial habitats is lacking. The identification of the new *Metopus* species provided an opportunity to investigate the existence and identities of such bacteria in this

![Figure 6](image_url)

**Figure 6** The classification and assemblage composition of digestion-resistant bacteria in the cytoplasm of *Metopus yantaiensis* n. sp. (A) A maximum likelihood (ML) tree based on 16S rRNA genes, showing the phylogenetic positions of the intracellular bacteria. The newly obtained sequences are highlighted in bold. The GTR + G + I model was used. GenBank accession numbers follow each species names. Bootstrap values lower than 50% and posterior probability lower than 0.5 were not shown. (B) Pie chart showing the composition of digestion-resistant bacteria associated with the ciliate host.

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anaerobic soil species. The DRB in \textit{M. yantaiensis} n. sp. are dominated by Alpha- and Gammaproteobacteria, which is consistent with previous studies on marine and freshwater protists (Farnelid et al. 2016; Gong et al. 2016; Martinez-Garcia et al. 2012; Pucciarelli et al. 2015). Hirakata et al. (2015) isolated a strain of endosymbiotic bacteria (family Clostridiaceae) from \textit{M. contortus} by anaerobic cultivation with antibiotics. Coincidently, a \textit{Clostridium}-like was also detected in our isolates of \textit{M. yantaiensis}, suggesting a tight association between \textit{Metopus} spp. and Clostridiales.

The bacterium \textit{Pseudomonas alcaligenes} found in this study is known to contain the secretion system type VI (T6SS), which might facilitate the survival of the intracellular bacterium and its communication with the eukaryotic host (Gong et al. 2016; Jani and Cotter 2010). Taken together, the study of DRB in the new soil ciliate species largely supports previous findings and the secretion system hypothesis for bacteria-protist associations.

\section*{TAXONOMIC SUMMARY}

Class Armophorea Lynn, 2004
Order Metopida Jankowski, 1980
Family Metopidae Kahl, 1927
Genus \textit{Metopus} Claparède and Lachmann, 1868

\textit{Metopus yantaiensis} n. sp.

\textbf{Diagnosis.} Size in vivo usually 65–105 \times 35–55 \mu m. Body ellipsoidal with nuclear apparatus in preoral dome. Cortical granules inconspicuous. Five perizonal and 18–21 widely spaced somatic kineties, three of which extend onto preoral dome. Three to five elongated caudal cilia. Adoral zone composed of about 21–29 polykinetids.

\textbf{Type locality.} Soil from an apple orchard near the city of Yantai, Shandong, China, 37°57'73"N, 120°66'51"E.

\textbf{Type material.} One holotype (reg. no. OUC-CN 2017.01.13.01) and two paratype slides (reg. nos. OUC-CN 2017.01.13.02 and OUC-CN 2017.01.13.03) with protargol-impregnated specimens have been deposited in the Laboratory of Protozoology, OUC, China. Another paratype slide (reg. no. NHMUK 2017.3.10.1) with protargol-impregnated specimens has been deposited in the Natural History Museum, London, U.K. The holotype and important paratype specimens have been marked by black ink circles on the coverslip.

\textbf{Gene sequence.} A sequence of the 18S rRNA gene of \textit{Metopus yantaiensis} n. sp. has been deposited in the GenBank database with the accession number KY432959.

\textbf{Etymology.} Named after the city in which it was discovered.

\textbf{Zoobank accession number.} urn:lsid:zoobank.org:act:33D4FB91-5A85-4376-9C9F-72AA0F9B7CF4.
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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Morphological information of sequenced Ato-

Pospira, Heterometopus, Metopus, and Parametopidium species.