



Morphology and Molecular Phylogeny of Four Trachelocercid Ciliates (Protozoa, Ciliophora, Karyorelictea) Found in Marine Coastal Habitats of Northern China, With Description of a New Genus, Two New Species and a New Combination

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The morphology of four trachelocercid ciliates, *Paratrachelocerca typica* gen. nov., spec. nov., *Trachelolophos monocaryon* (Dragesco, 1965) comb. nov. (original combination: *Tracheloraphis monocaryon* Dragesco, 1965), *Tracheloraphis katzae* spec. nov., and *Tracheloraphis colubis* (Kahl, 1933) Xu et al., 2011 were studied in live and protargol-stained specimens. All samples were isolated from the intertidal zone of sandy beaches at Qingdao, China. The new genus *Paratrachelocerca* can be distinguished from other trachelocercid genera mainly by the three circumoral kineties each composed of a row of dikinetids and the absence of a brosse or ciliary tuft in the oral cavity. The detailed investigation on the poorly described *Tracheloraphis monocaryon* (Dragesco, 1965) reveals that its oral infraciliature includes one uninterrupted circumoral kinety and a conspicuous ciliary tuft in the center of the oral cavity, which is consistent with the genus *Trachelolophos* rather than *Tracheloraphis*. Therefore, this species is transferred to *Trachelolophos* as *Trachelolophos monocaryon* (Dragesco, 1965) comb. nov. *Tracheloraphis katzae* spec. nov. can be recognized by the combination of its minute brownish cortical granules and 9–15 somatic kineties. The small subunit (SSU) rDNA of each species was sequenced for the first time. Phylogenetic analyses of the SSU rDNA show that *Paratrachelocerca typica* gen. nov., spec. nov. clusters with *Apotrachelocerca arenicola* (Kahl, 1933) Xu et al., 2011 in a group that is sister to all other trachelocercids.

Keywords: infraciliature, SSU rDNA, ciliates, Karyorelictea, Trachelocercidae

INTRODUCTION

The family Trachelocercidae Kent, 1881 is the largest family within the class Karyorelictea Corliss, 1974 and all its members inhabit marine sandy sediments in intertidal zones (Al-Rasheid, 1996, 1997, 1998, 2001; Foissner and Dragesco, 1996a; Al-Rasheid and Foissner, 1999; Song et al., 2009; Hu et al., 2019). Since the first species was described over 200 years ago, about 80 nominal species of trachelocercids have been reported (Carey, 1992; Xu et al., 2011a, 2014; Yan et al., 2015, 2016). However, only a limited number of detailed studies of trachelocercids have been carried out using modern methods such as protargol staining, scanning electron microscopy and phylogeny analyses (Foissner and Dragesco, 1996a,b; Dragesco, 1997, 1999; Foissner, 1997; Xu et al., 2012; Yan et al., 2019) and most species are known only from live observations (Dragesco, 1960; Raikov et al., 1975; Wilbert, 1986; Carey, 1992). Moreover, the molecular data of trachelocercids remain scarce.

In the present study, the morphological data of four trachelocercids isolated from marine coastal habitats at Qingdao, China, are documented based on observations of specimens *in vivo* and following protargol staining. In addition, the SSU rDNA sequence of each species is provided and phylogenetic analyses are performed to assess their evolutionary relationships. *Paratrachelocerca* gen. nov. is assigned to the family Trachelocercidae based on both morphological and molecular information.

MATERIALS AND METHODS

Sample Collection, Observation, and Identification

Paratrachelocerca typica gen. nov., spec. nov. and *Tracheloraphis colubis* (Kahl, 1933) Xu et al., 2011 were collected from the intertidal zone of Silver Beach, Qingdao (35°55'09"N, 120°11'55"E) on March 11, 2013 and May 27, 2019, respectively. The water temperature on each day of sampling was 11 and 23°C, respectively, and the salinity was about 27‰. *Trachelolophos monocaryon* (Dragesco, 1965) comb. nov. and *Tracheloraphis katzae* spec. nov. were both collected on June 24, 2019, from the intertidal zone of the No. 1 Bathing beach, Qingdao (36°03'24"N, 120°20'32"E) where the water temperature was 26°C and the salinity was about 30‰ (Figure 1). For each sample, the top 5 cm of sand or sediment along with seawater from the site was collected. Ciliates were extracted from the sediment using the method from Uhlig (1968). In brief, the sand or sediment was placed in a plastic tube (4.5 cm diameter and 10 cm long, at one end of which was a tightly fitting nylon gauze (mesh size 80–90 μm)). The depth of the sediment in the tube was 5 cm. Finely crushed ice was added to fill the remainder of the tube. A glass culture dish containing about 20 ml filtered seawater was placed under the tube so that the nylon gauze was barely in contact with the seawater surface. Ciliates in the sand/sediment sample migrating downwards

to escape the advancing front of meltwater were collected in the culture dish.

Cells were isolated and observed *in vivo* using bright field and differential interference contrast (DIC) microscopy (Olympus BX 53). The infraciliature was revealed using the protargol staining method (Wilbert, 1975). Counts, measurements, and drawings of stained specimens were performed at 1,000 × magnification. Terminology and systematics mainly follow Foissner (1996) and Lynn (2008), respectively.

DNA Extraction, PCR Amplification, and Gene Sequencing

DNA extraction, PCR amplification, and SSU rDNA sequencing of the four species were performed according to Wang et al. (2020). For each species, we extracted total genomic DNA from single cells using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The primers 82F (5'-GAA ACT GCG AAT GGC TC-3') (Jerome et al., 1996), 18S-F (5'-AAC CTG GTT GAT CCT GCC AGT-3'), and 18S-R (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') were used to amplify the SSU rDNA (Medlin et al., 1988). PCR amplification was carried out according to Wang et al. (2019, 2020) and PCR products were then sequenced bidirectionally by the Tsingke Biological Technology Company (Beijing, China).

Phylogenetic Analyses

Using MAFFT implemented in GUIDANCE¹ with default parameters (Penn et al., 2010), the newly generated SSU rDNA sequences of the four species were aligned with 54 other sequences of karyorelictean and heterotrich (outgroup) species downloaded from NCBI GenBank. The resulting alignments were manually refined by trimming both ends with Bioedit v.7.0.5 (Hall, 1999) and the final alignments were 1,802 bp.

Maximum likelihood (ML) and Bayesian inference (BI) analyses were both carried out using the CIPRES Science Gateway v.3.3² (Stamatakis, 2014). ML bootstrapping analysis was performed online with 1,000 replicates using RAXML-HPC2 on XSEDE v.8.2.12 (Stamatakis et al., 2008) and the GTRGAMMA model. BI analysis was performed with MrBayes on XSEDE v.3.2.7a (Ronquist et al., 2012) using the GTR + I + G model selected by MrModeltest v.2.2 (Nylander, 2004). The chain length for our analysis was 1,000,000 generations with trees sampled every 100 generations, the first 25% of which were discarded as burn-in. MEGA v.5.0 (Tamura et al., 2011) was used to visualize the phylogenetic tree topology.

Topology Testing

The phylogenetic relationships among different taxa within Karyorelictea were assessed using the approximately unbiased (AU) test (Shimodaira, 2002). Two constrained ML trees were generated by RAXML v8.2.10 (Stamatakis, 2014) with the enforced constraints (Table 1) and their topologies were compared with those of the best unconstrained ML trees

¹<http://guidance.tau.ac.il/ver2/>

²<http://www.phylo.org/portal2>

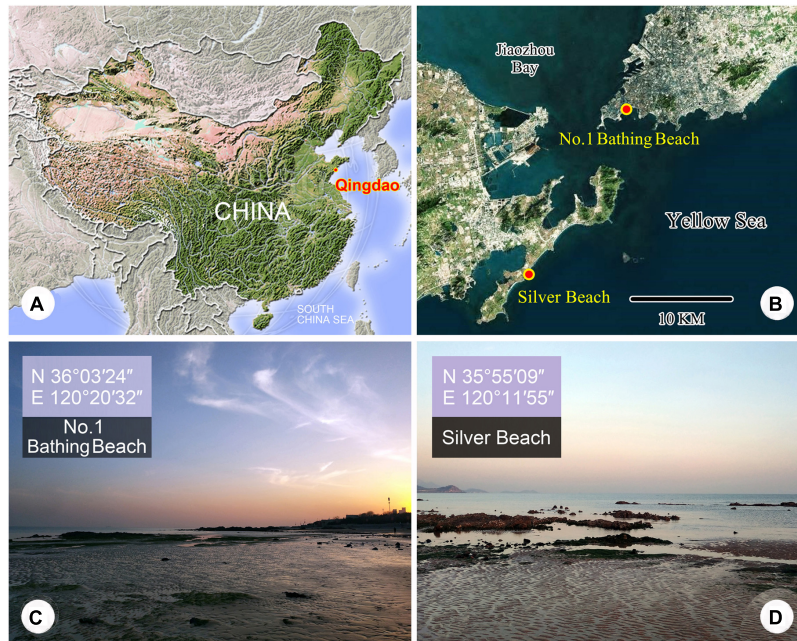


FIGURE 1 | (A–D) Maps and photographs of the sample sites. **(A)** Location of Qingdao. **(B)** The dots indicates the locations of the two sample sites. **(C)** The intertidal zone of the No. 1 Bathing Beach. **(D)** The intertidal zone of Silver Beach.

TABLE 1 | Approximately Unbiased test results based on the SSU rDNA data.

Topology constraints	Log-likelihood (-lnL)	AU test
Unconstrained	-13786.067660	0.935
<i>Tracheloraphis</i>	-13954.259391	7.00E-14
<i>Trachelocerca</i>	-13802.664146	0.065

The topology constraints column refers to proposed taxonomic groups that were tested for monophyly through the approximately unbiased test (AU). Rejected monophyly ($p < 0.05$) is highlighted in gray.

implemented in CONSEL (Shimodaira and Hasegawa, 2001). Internal relationships in the constrained group and among the remaining taxa were unspecified.

ZooBank Registration

ZooBank registration number of present work: urn:lsid:zoobank.org:pub:913C263C-56A7-40AC-9885-E06DC530A6D8.

RESULTS AND DISCUSSION

Class Karyorelictea Corliss, 1974

Order Protostomatida Small & Lynn, 1985

Family Trachelocercidae Kent, 1881

Genus *Paratrachelocerca* gen. nov.

Diagnosis

Trachelocercidae with three circumoral kineties each composed of a row of dikinetids. No brosse or ciliary tuft in oral cavity. Marine habitat.

Etymology

The new genus name is dedicated to the eminent ciliatologist, Prof. Wilhelm Foissner, Universität Salzburg, Austria, in recognition of his significant contributions to the study of ciliates.

Type Species

Paratrachelocerca typica spec. nov.

Discussion

Paratrachelocerca gen. nov. possesses all the diagnostic characters of trachelocercids, i.e., elongate body shape with distinct “head” and “neck” and somatic ciliature covering the body apart from longitudinal glabrous zone that is bordered by a “bristle-like” kinety. Therefore, *Paratrachelocerca* gen. nov. undoubtedly belongs to the family Trachelocercidae. The shape and structure of the oral ciliature is the main character for generic classification (Foissner and Dragesco, 1996a,b; Foissner and Al-Rasheid, 1999). *Paratrachelocerca* gen. nov. possesses three circumoral kineties each composed of a row of dikinetids. Consequently, it can be easily separated from its most closely related genera such as *Apotrachelocerca* Xu et al., 2011, which has two rows of uninterrupted circumoral kineties, *Trachelocerca* Ehrenberg, 1840, which has a single uninterrupted circumoral kinety composed of dikinetids, and *Prototrachelocerca* Foissner, 1996 has two rows of circumoral kineties interrupted by short brosse kineties (Table 2). It is noteworthy that no anterior or posterior secant system is present in *Paratrachelocerca typica* gen. nov. spec. nov., which is similar to *Apotrachelocerca arenicola* (Kahl, 1933) Xu et al., 2011. However, the presence/absence of the

TABLE 2 | Distinction among genera in trachelocercid karyorelictids.

Genus	Character					Source
	Brosse	Ciliary tuft in oral cavity	Circumoral kinety	Secant system		
<i>Paratrachelocerca</i> gen. nov.	–	–	Three rows and uninterrupted	Left		This study
<i>Trachelolophos</i>	–	Present	One row and uninterrupted	Left		Foissner and Dragesco (1996a)
<i>Tracheloraphis</i>	Present	–	One row and interrupted	Left		Dragesco (1960)
<i>Prototrachelocerca</i>	Present	–	Two rows and interrupted	Left		Foissner (1996)
<i>Kovalevaia</i>	Present	–	One row and uninterrupted	Left		Foissner (1997)
<i>Sultanophrys</i>	Present	–	One row and interrupted	Right		Foissner and Al-Rasheid (1999)
<i>Apotrachelocerca</i>	–	–	Two rows and uninterrupted	Left		Xu et al. (2011b)
<i>Trachelocerca</i>	–	–	One row and uninterrupted	Left		Ehrenberg (1840)

–, Absent.

The genera in bold are described in present study.

anterior or posterior secant system is not considered as a genus-level character for trachelocercid classification.

***Paratrachelocerca typica* spec. nov.** (Figures 2, 3 and Table 3)

Diagnosis

Extended cells *in vivo* about 400–800 $\mu\text{m} \times 15\text{--}25 \mu\text{m}$; body ribbon-like and flattened; head dominant and black in color, distinguished from trunk; usually 13–24 macronuclei; 7 ciliary rows on right side of cell; left side unciliated except for bristle kinety; glabrous stripe

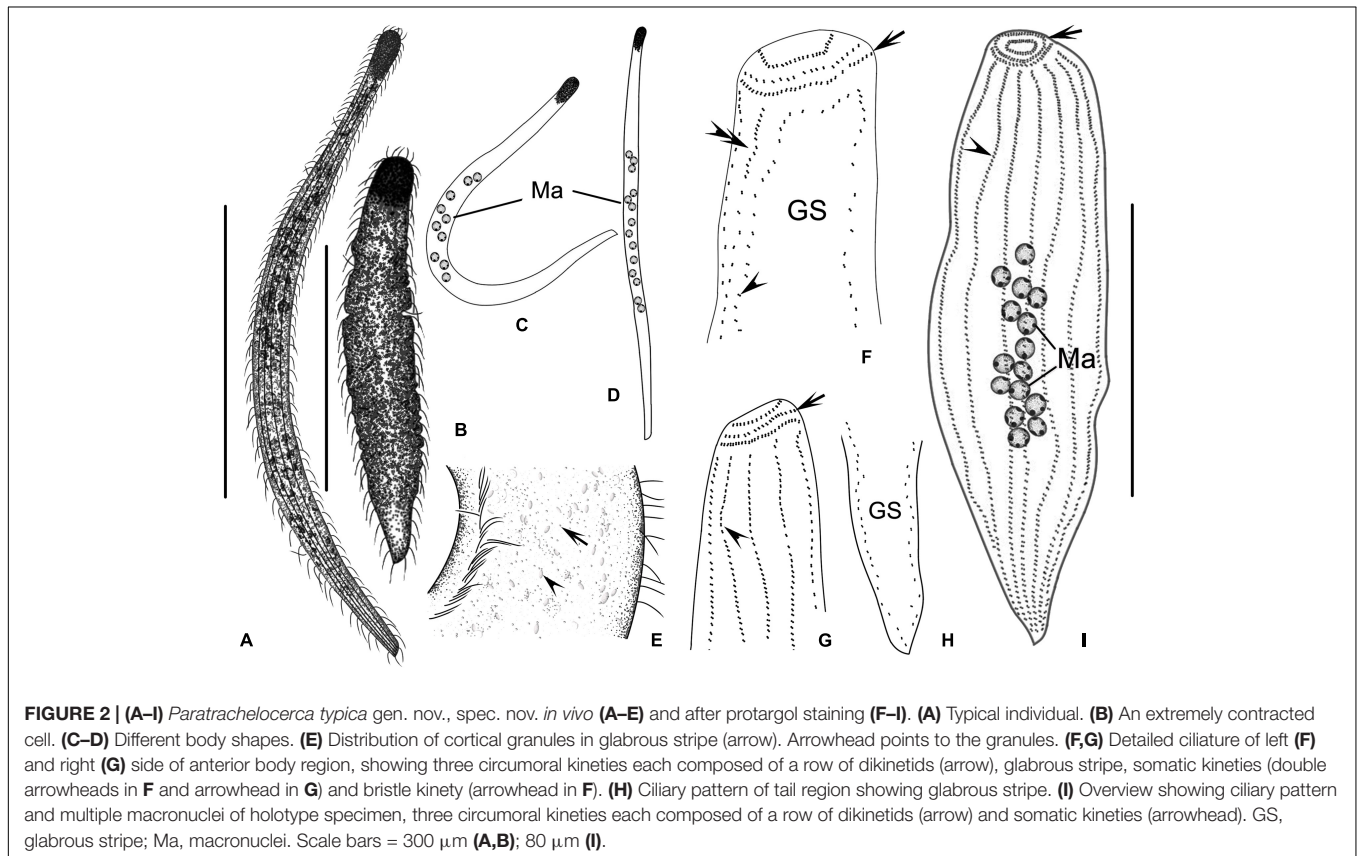
as wide as trunk; no anterior or posterior secant system on either side of glabrous stripe; cortical granules minute and colorless.

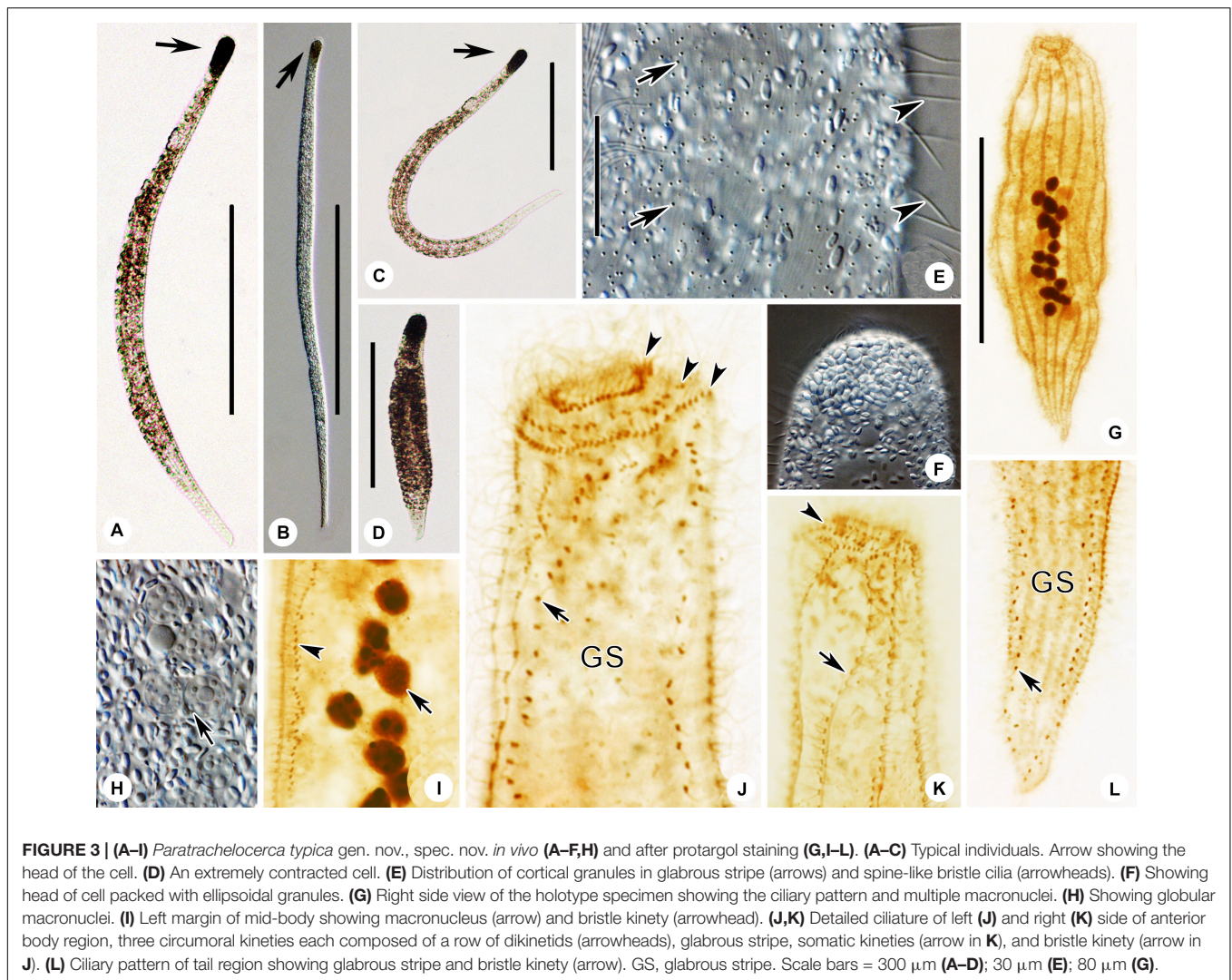
Type Locality

The intertidal zone of Silver Beach, Qingdao (35°55′09″N, 120°11′55″E), China (Figure 1).

Type Specimens

A protargol-stained slide containing the holotype specimen marked with an ink circle is deposited in the Laboratory





of Protozoology, Ocean University of China, Qingdao, China (No. YY2013031107).

Etymology

This species-group name *typica* means that it is the representative species of this genus.

Morphological Description

Cells *in vivo* 400–800 μm \times 15–25 μm , flexible and contractile (Figures 2A,B, 3A–D); ribbon-like flattened (up to 3:1) including oral area (Figures 2A,C,D, 3A–C). Width almost constant throughout anterior three-quarters of cell; head region dominant; no distinct neck; posterior quarter of body narrowed (Figures 2A,C,D, 3A–C). At low magnification, anterior of cell often dark grayish due to multiple inclusions (Figure 3F). Cortical granules colorless, about 0.5 μm in diameter, sparsely scattered between ciliary rows and in glabrous stripe (Figures 2E, 3E). Thirteen to 26 macronuclei, 6–9 μm in diameter, arranged in a longitudinally oriented group in mid-body region (Figures 2C,D, 3G and Table 3). Macronuclei

containing many large chromatin aggregates, possibly nucleoli (Figures 2I, 3H,I).

Locomotion by gliding sluggishly along bottom of Petri dish.

Infraciliature consists of dikinetids (Figure 2F–I). Only right side ciliated with seven somatic kineties. Left side occupied by glabrous stripe. Cilia about 8 μm long (Figure 3G). No anterior or posterior secant system on either side of glabrous stripe (Figures 2E,H, 3I,J,L). Glabrous stripe bordered by a bristle kinety composed of one row of dikinetids (Figures 2F,H, 3I,J,L). Three circumoral kineties each composed of an uninterrupted row of obliquely oriented and narrowly spaced dikinetids (Figures 2E,G, 3J,K).

Genus *Trachelolophos* Foissner & Dragesco, 1996

Trachelolophos monocaryon (Dragesco, 1965) **comb. nov.** (Figures 4, 5 and Table 3)

(original combination: *Tracheloraphis monocaryon* Dragesco, 1965)

Dragesco (1965) described this species under the name of *Tracheloraphis monocaryon*, based solely on live observations. Thus, this form remained largely unknown for over half a

TABLE 3 | Morphometric data for *Paratrachelocerca typica* gen. nov., spec. nov. (first line), *Trachelolophos monocaryon* (Dragesco, 1965) comb. nov. (second line), *Tracheloraphis katzae* spec. nov. (third line), and *Tracheloraphis colubis* (fourth line).

Characters	Min	Max	Mean	SD	CV	n
Body length in μm	142	329	198.9	53.1	26.7	24
	135	430	279.6	85.0	30.4	25
	104	255	168.5	39.5	23.4	25
	120	308	174.0	38.3	22.0	25
Body width in μm	20	78	46.4	14.2	30.5	24
	42	150	78.6	28.7	36.5	25
	20	53	30.1	8.3	76.5	25
	30	105	62.4	20.5	32.9	25
Somatic kineties, number	7	7	7.0	0	0	21
	30	44	36.7	3.7	10.2	25
	9	15	11.8	1.4	11.8	25
	17	27	21.8	2.6	11.7	23
Macronuclei in single nuclear group, number	–	–	–	–	–	–
	4	4	4.0	0	0	25
	4	4	4.0	0	0	25
	4	4	4.0	0	0	25
Macronuclei in strand, number	13	26	17.0	3.2	19.1	22
	–	–	–	–	–	–
	–	–	–	–	–	–
	–	–	–	–	–	–
Micronuclei in single nuclear group, number	–	–	–	–	–	–
	2	2	2.0	0	0	25
	2	2	2.0	0	0	25
	2	2	2.0	0	0	25
Nuclear group length	–	–	–	–	–	–
	19	33	25.4	4.1	16.3	25
	9	14	10.8	1.4	12.6	25
	9	17	11.4	2.2	18.9	25

All data are based on protargol-stained specimens and morphometric data of *Trachelolophos monocaryon* (Dragesco, 1965) comb. nov. and *Tracheloraphis katzae* spec. nov. are based on Qingdao population. CV, coefficient of variation (%); Mean, arithmetic mean; n, number of examined specimens. “–” indicates data unavailable or not applicable.

century due to the lack of knowledge of the infraciliature. Observations of both live and silver-stained specimens of the present isolate indicates that this taxon should be assigned to the genus *Trachelolophos*. Therefore, a new combination is suggested and a redescription and improved diagnosis are supplied.

Improved Diagnosis

Extended cells 500–1,000 μm \times 40–70 μm *in vivo* with inconspicuous dark head and a rounded posterior end. Thirty to 44 somatic kineties. Glabrous stripe narrow, about the width occupied by 2–3 somatic kineties. Four macronuclei and two micronuclei in a single group. Cortical granules grayish and ellipsoidal, ca. 0.5 μm \times 0.8 μm , densely distributed.

Deposition of Voucher Materials

A voucher slide with protargol-stained specimens has been deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, China (registration number: MMZ2019062406).

Redescription

Extended cells 500–1,000 μm \times 40–70 μm *in vivo*; body cylindrical or rod-like, flexible and contractile (Figures 4A–C, 5A–D); 30–44 somatic kineties; neck and tail indistinctly separated from trunk, head triangular, posterior end of tail rounded (Figures 4A–C,H, 5A–D and Table 3). Cortical granules ellipsoidal, ca. 0.5 μm \times 0.8 μm , grayish in bright field at high magnifications; densely packed (but non-grouped) between somatic kineties and in glabrous stripe (Figures 4D, 5E). Cytoplasm colorless and transparent, packed with cytoplasmic granules, ellipsoidal, 1–3 μm long and colorless (Figures 5F,G).

Locomotion by gliding on substrate, winding between sand grains and organic debris.

Entire infraciliature consisting of dikinetids (Figures 4H, 5K). Cilia about 10 μm long *in vivo* and arranged in longitudinal rows. Oral infraciliature consists of a single uninterrupted circumoral kinety and a conspicuous ciliary tuft located in center of oral cavity (Figures 4F–H, 5I,L). Glabrous stripe very narrow, width about equal to the gap between two adjacent somatic kineties, bordered by irregularly spaced bristle kinety (Figures 4H, 5J). Anterior and posterior secant systems formed on left side of glabrous stripe and some kineties also abut to bristle kinety (Figures 4H, 5J). Four macronuclei (in which crystals are sometimes present) and two micronuclei in a single group (Figures 4E, 5H,M).

Discussion

This organism was first reported by Dragesco (1965) under the name *Tracheloraphis monocaryon* and the original description was based solely on a rather schematic figure and a short description of the cell *in vivo*. The Qingdao population corresponds closely with the original population in several key characters such as the narrow glabrous stripe, the single nuclear group composed of four macronuclei and two micronuclei and the possession of about 40 somatic kineties. Therefore, we consider them to be conspecific. Before transferring this species to *Trachelolophos*, we compare the Qingdao isolate to all four species of *Trachelolophos*.

The four congeners can all be clearly distinguished from the new isolate by body shape, body length, the number of somatic kineties and the number of nuclei or nuclear groups. *Trachelolophos filum* (Dragesco & Dragesco-Kernéis, 1986) Foissner & Dragesco, 1996 differs from the new isolate in having fewer somatic kineties on the trunk (26–35 vs. 30–44) and 4–16 nuclear groups, each with usually two macronuclei and a micronucleus, whereas the Qingdao isolate has only one nuclear group consisting of four macronuclei and two micronuclei (Dragesco and Dragesco-Kernéis, 1986; Foissner and Dragesco, 1996a). *Trachelolophos gigas* Foissner & Dragesco, 1996 differs from *T. monocaryon* in possessing a much longer body (2,000 μm vs. 500–1,000 μm), more somatic kineties on the trunk (52–71 vs. 30–44), and in the number and arrangement of its macronuclei (17–33 forming a strand vs. four in one nuclear group) (Foissner and Dragesco, 1996a). *Trachelolophos binucleatus* Yan et al., 2016 differs from *T. monocaryon* in having fewer somatic kineties

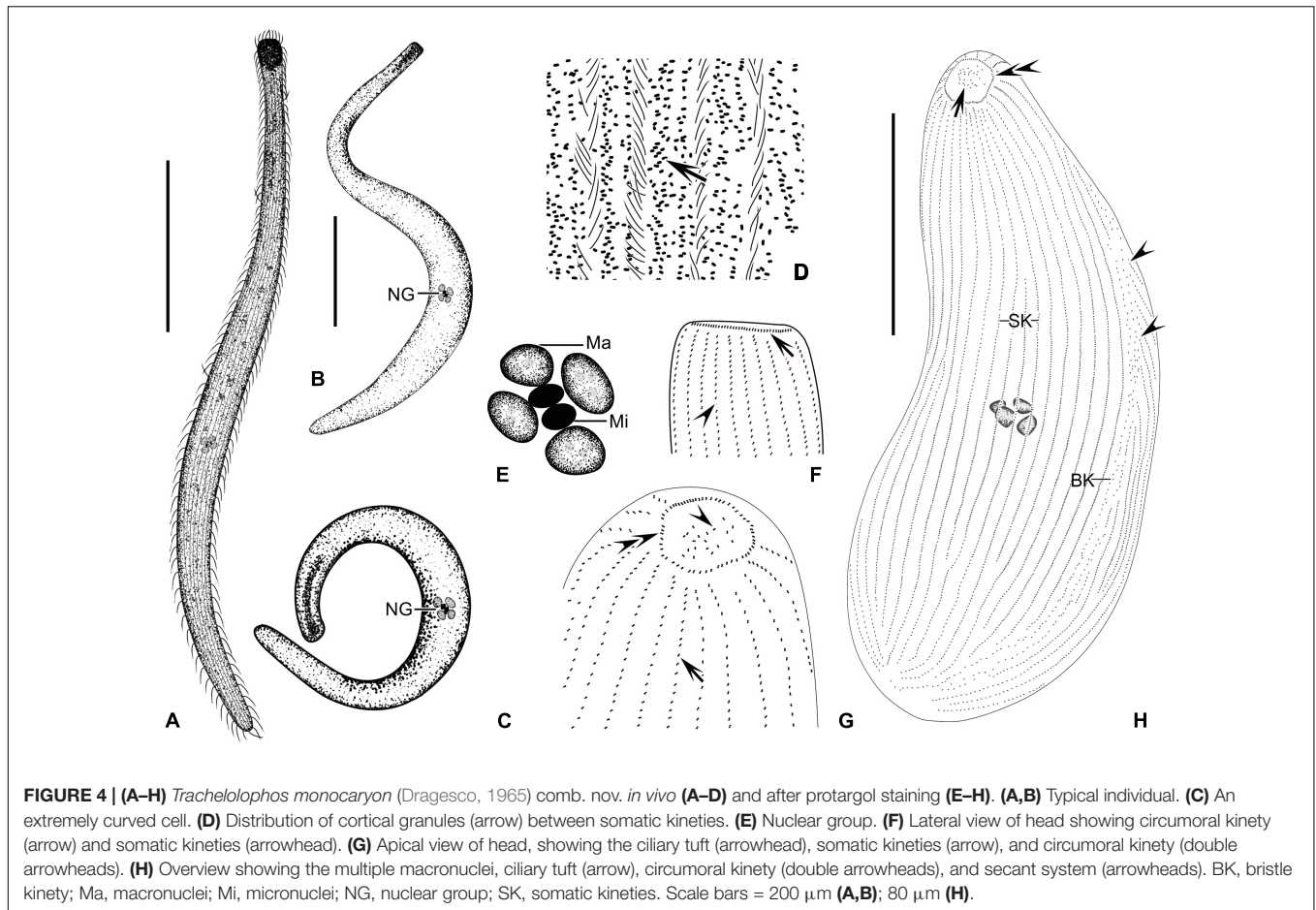


FIGURE 4 | (A–H) *Trachelolophos monocaryon* (Dragesco, 1965) comb. nov. *in vivo* (A–D) and after protargol staining (E–H). (A,B) Typical individual. (C) An extremely curved cell. (D) Distribution of cortical granules (arrow) between somatic kineties. (E) Nuclear group. (F) Lateral view of head showing circumoral kinety (arrow) and somatic kineties (arrowhead). (G) Apical view of head, showing the ciliary tuft (arrowhead), somatic kineties (arrow), and circumoral kinety (double arrowheads). (H) Overview showing the multiple macronuclei, ciliary tuft (arrow), circumoral kinety (double arrowheads), and secant system (arrowheads). BK, bristle kinety; Ma, macronuclei; Mi, micronuclei; NG, nuclear group; SK, somatic kineties. Scale bars = 200 μm (A,B); 80 μm (H).

on the trunk (17–26 vs. 30–44) and fewer macronuclei (2 vs. 4) (Foissner and Dragesco, 1996a). *Trachelolophos quadrinucleatus* Yan et al., 2016 differs from *T. monocaryon* in possessing a longer body (1,100–1,400 μm vs. 500–1,000 μm) and a wedge-shaped (vs. rounded) posterior body end (Yan et al., 2016). Given these distinctions, the validity of *T. monocaryon* as a distinct species within the genus *Trachelolophos* is strongly supported.

Genus *Tracheloraphis* Dragesco, 1960

Tracheloraphis katzae spec. nov. (Figures 6, 7 and Table 3)

Diagnosis

Size *in vivo* 400–800 μm \times 20–30 μm ; 9–15 somatic kineties on trunk; head dark and conspicuous, trunk widened, tail narrowed; single nuclear group composed of four macronuclei and two micronuclei; glabrous stripe as wide as trunk; cortical granules brownish, ellipsoidal, about 0.5 μm \times 0.2 μm , densely distributed.

Type Locality

The intertidal zone of the No. 1 Bathing beach, Qingdao (36°03'24"N, 120°20'32"E), China, where the water temperature was 26°C and the salinity was about 30‰ (Figure 1).

Type Specimens

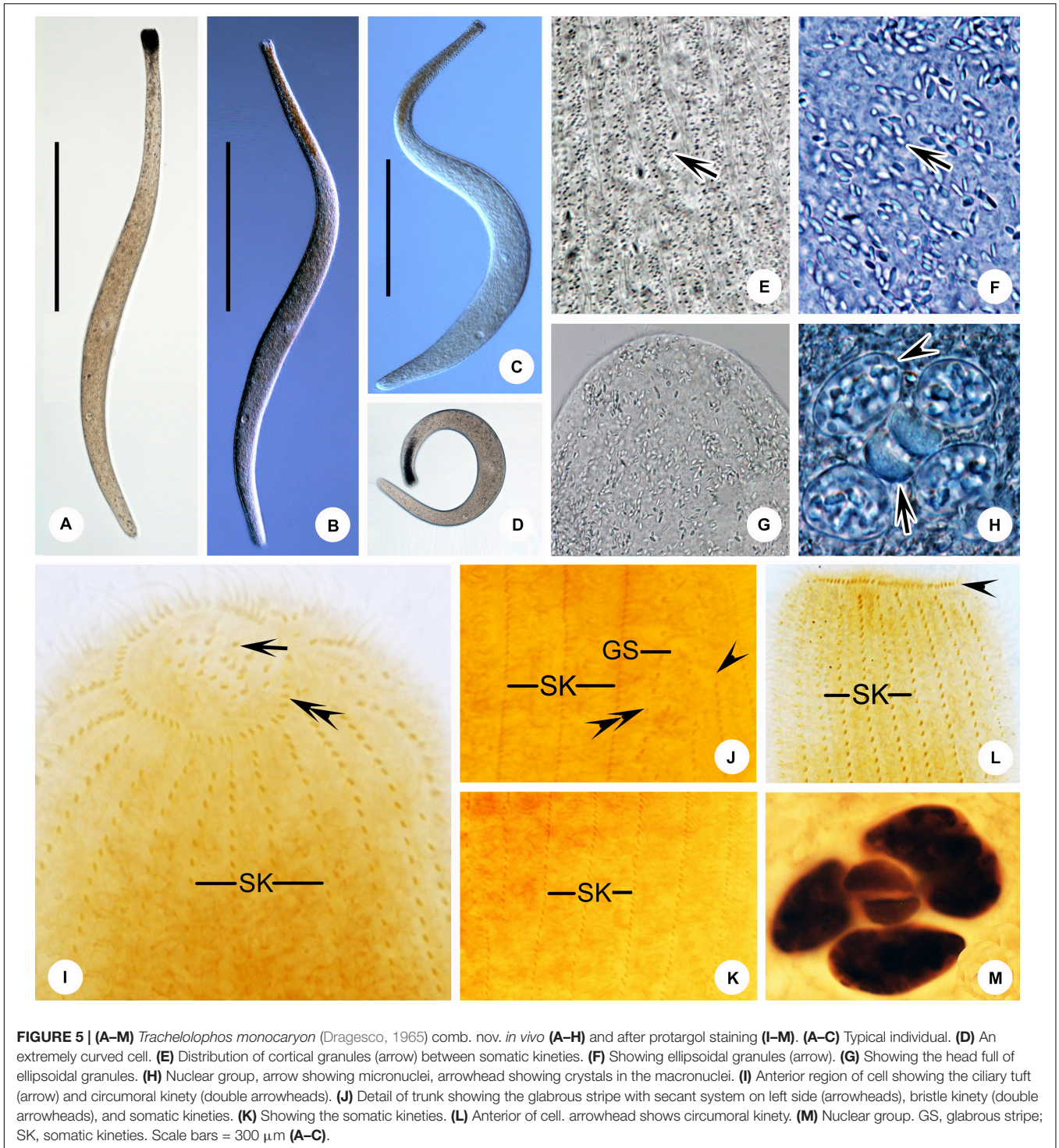
A protargol slide containing the holotype specimen marked with an ink circle is deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, China (No. MMZ2019062407).

Etymology

We dedicate this new species to our eminent colleague, Prof. Laura Katz, Smith College, United States, in recognition of her great contributions to ciliate research.

Description

Fully extended cells about 600 μm \times 25 μm *in vivo*; body flexible and flattened, ribbon-like, with claviform head and pointed tail, trunk region conspicuously widened (Figures 6A–C, 7A–C). Trunk dark at low magnification, neck and tail portions transparent due to lack of inclusions (Figures 6A–C, 7A). Globular granules clustering in mid-body region (Figure 7F). Single nuclear group located in center of trunk, containing four macronuclei, 5–7 μm in diameter, in which there are some crystals (Figure 7G), and two micronuclei, 2–3 μm in diameter (Figures 6F, 7G,L). Brownish cortical granules, ellipsoidal, ca. 0.2 μm \times 0.5 μm , densely distributed between ciliary rows and in glabrous stripe (Figures 6E, 7D,E).



Locomotion by gliding between sand grains and organic debris. Cells surface densely ciliated (Figures 6I, 7H). Glabrous stripe about as wide as trunk (Figures 6H, 7I). Entire infraciliature consisting of dikinetids with cilia ca. 10 μm long (Figures 6A,E,I). Five to ten somatic kineties on head, 9–15 on trunk. Anterior and posterior secant systems on left side of glabrous stripe, some kineties abut to bristle kinety

(Figures 6D,H, 7I,K,M). Oral ciliature composed of single-rowed circumoral kinety, interrupted by two inserted crosses (Figures 6G,H, 7I,J).

Discussion

As shown in Figure 8 and Table 4, the current new species should be compared with its most similar congeners (Figure 8

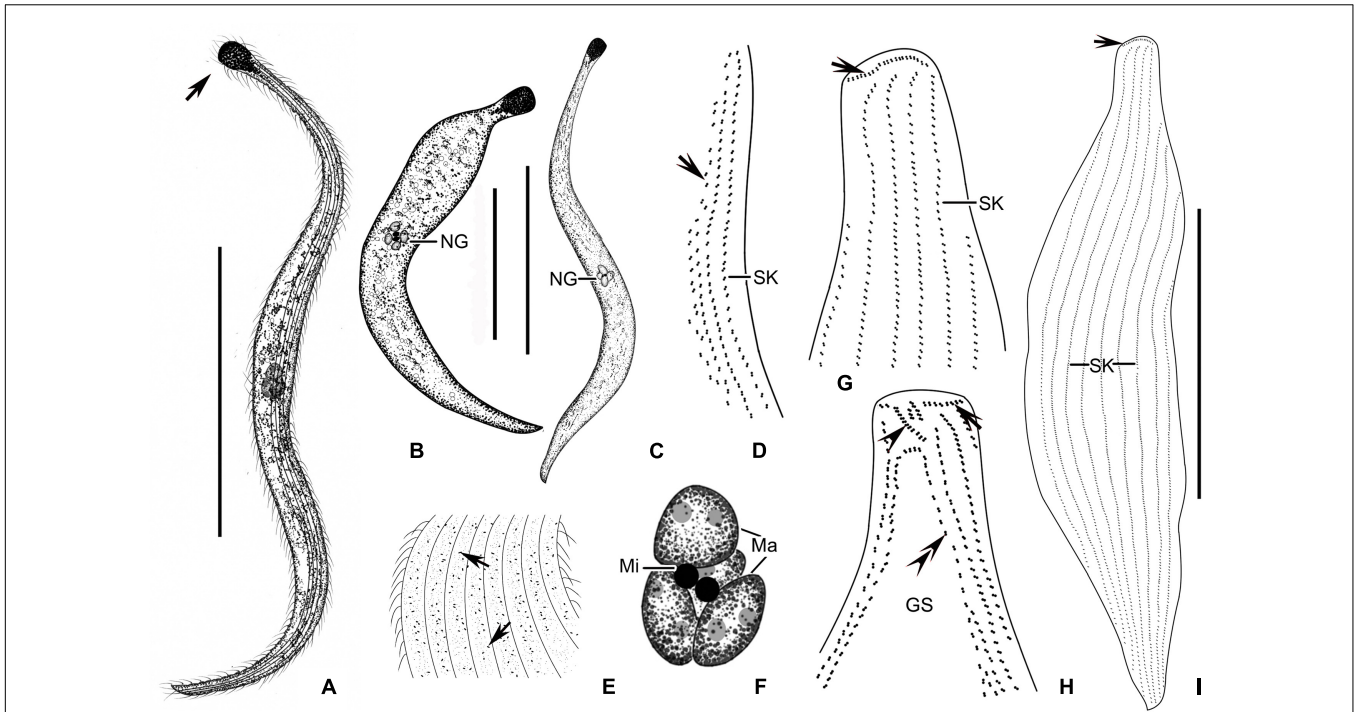


FIGURE 6 | (A–I) *Tracheloraphis katzae* spec. nov. *in vivo* (A–C,E) and after protargol staining (D,F–I). (A–C) Typical individual. Arrow showing the head of the cell. (D) Ciliary pattern of bristle kinety (arrow) and somatic kinety. (E) Distribution of cortical granules (arrows) between somatic kineties. (F) Nuclear group. (G,H) Detailed ciliature of left (H) and right (G) side of anterior body region. Arrowhead in (H) showing brosse, arrow showing circumoral kinety, double arrowheads showing bristle kinety. Arrowhead in (G) showing circumoral kinety. (I) Right view of the holotype specimen showing the ciliary pattern, arrow depicts circumoral kinety. GS, glabrous stripe; SK, somatic kineties. Scale bars = 200 μm (A–C); 70 μm (I).

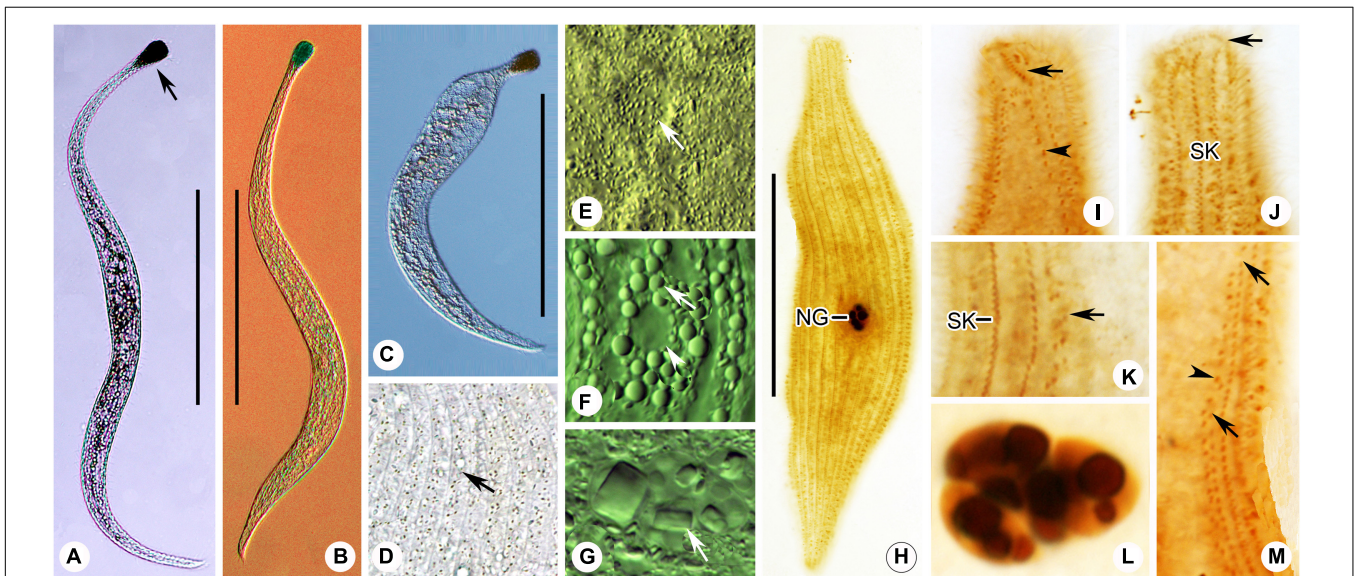


FIGURE 7 | (A–M) *Tracheloraphis katzae* spec. nov. *in vivo* (A–G) and after protargol staining (H–M). (A–C) Typical individual. Arrow showing the head of the cell. (D) Distribution of cortical granules (arrow) between somatic kineties. (E) Distribution of cortical granules in glabrous stripe (arrow). (F) Arrow showing globular granules in cell. Arrowhead showing a vacuole. (G) Nuclear group. Arrow showing crystals in the macronuclei. (H) Right view of the holotype specimen showing the ciliary pattern and multiple macronuclei. (I,J) Detailed ciliature of left (I) and right (J) side of anterior body region. Arrow in I shows brosse, arrowhead showing bristle kinety. Arrow in (J) showing circumoral kinety. (K) Arrow showing somatic kineties. (L) Nuclear group. (M) Arrows showing secant system on left side of glabrous stripe. Arrowhead showing bristle kinety. NG, nuclear group; SK, somatic kineties. Scale bars = 200 μm (A–C); 70 μm (H).

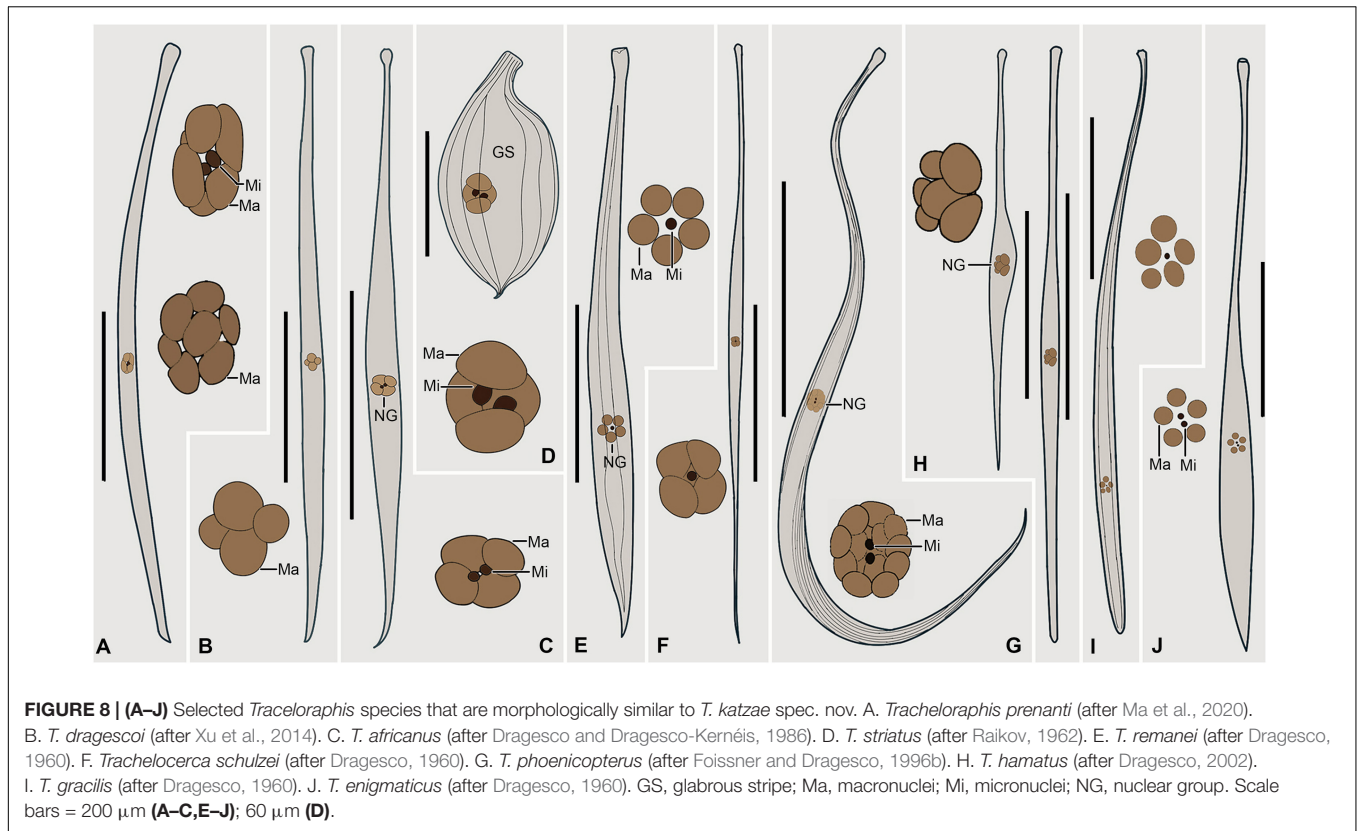


TABLE 4 | Comparison of *Tracheloraphis katzae* spec. nov. (in bold) with morphologically similar congeners.

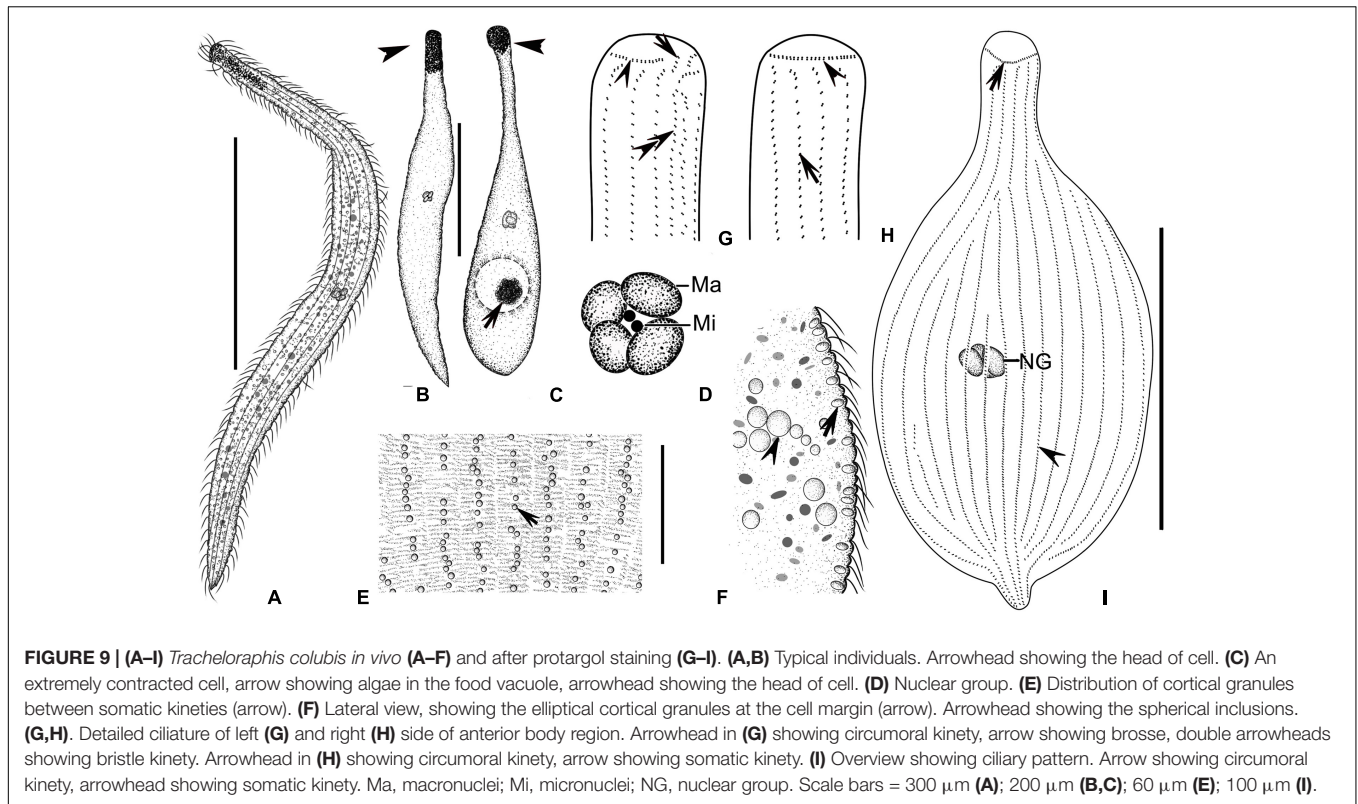
Species	Body length (μm)	SK (n)	Ma (n)	Source
<i>Tracheloraphis katzae</i> spec. nov.	400–800	9–15	4	This study
<i>Tracheloraphis phoenicopterus</i>	1,000–1,500	23–27 19–21	6–12	Foissner and Dragesco (1996b)
<i>Tracheloraphis prenanti</i>	400–2,000	14–26	4–10	Ma et al. (2020)
<i>Tracheloraphis dragescoi</i>	600–1,000	14–22	4	Xu et al. (2014)
<i>Tracheloraphis hamatus</i>	500–900	10–14	3–6	Dragesco (2002)
<i>Tracheloraphis africanus</i>	700	17 or 18	4	Dragesco and Dragesco-Kernéis (1986)
<i>Tracheloraphis gracilis</i>	400–800	12 or 13	4–6	Dragesco (1960)
<i>Tracheloraphis enigmaticus</i>	600	–	5	Dragesco (1960)
<i>Tracheloraphis striatus</i>	500–700	12–14	4	Raikov (1962)
<i>Tracheloraphis remanei</i>	1,000	–	5 or 6	Dragesco (1960)
<i>Trachelocerca schulzei</i>	650	12 or 13	4	Dragesco (1960)

Ma, number of macronuclei; SK, number of somatic kineties; –, no data available.

and **Table 4**). *Tracheloraphis prenanti* Dragesco, 1960 and *T. phoenicopterus* (Cohn, 1866) Dragesco, 1960 resemble *T. katzae* spec. nov. in body shape and the width of the glabrous stripe. They can be distinguished from the latter, however, by their greater number of somatic kineties (14–26, 19–27, vs. 9–15) and macronuclei in the nuclear group (6–12, 4–10, vs. 4). Features of cortical granules can further separate three species. Cortical granules of *T. prenanti* are colorless, globular and about 0.5 μm in diameter, and in *T. phoenicopterus* they are ellipsoidal, yellowish, and 0.6 μm \times 1.2 μm , whereas, in *T. katzae* spec. nov. they are ellipsoidal, brownish, and 0.2 μm \times 0.5 μm (**Figures 8A,G**; Foissner and Dragesco, 1996b; Ma et al., 2020).

Tracheloraphis dragescoi Xu et al., 2014 has a similar body size and number of macronuclei as *T. katzae* spec. nov., but it can be separated from the latter by its oval cortical granules (colorless, about 0.2 μm \times 1 μm vs. brownish, about 0.2 μm \times 0.5 μm) and in having 14–22 (vs. 9–15) somatic kineties (**Figure 8B**; Xu et al., 2014).

Tracheloraphis hamatus Wright, 1982 resembles the novel form in body size, width of the glabrous stripe and the number of macronuclei. It can be distinguished from the latter, however, by having a different type of cortical granules (globular, less than 0.5 μm in diameter vs. ellipsoidal, about 0.2 μm \times 0.5 μm) (**Figure 8H**).



Tracheloraphis africanus Dragesco, 1965 resembles *T. katzae* spec. nov. in body size and the number of macronuclei. However, it differs from the latter by the absence (vs. presence) of cortical granules although according to Dragesco (1965) the 3 μm long rod-shaped “granules” scattered in the superficial cytoplasm may be bacteria (Figure 8C).

Tracheloraphis gracilis Dragesco, 1960 has a similar number of macronuclei compared to *Tracheloraphis katzae* spec. nov. and both species have oval cortical granules, but it can be separated from the latter by its tail which has a rounded (vs. wedge-shaped) end (Figure 8I; Dragesco, 1960).

Based on the original illustration, the nuclear apparatus of *Tracheloraphis enigmaticus* Dragesco, 1960 consists of five macronuclei and two micronuclei, but these are not clustered in a nuclear group as they are in *T. katzae* spec. nov. Furthermore, the figure of *T. enigmaticus* shows that it has short subuliform tail, whereas the new species has a narrow tail with a wedge-shaped end (Figure 8J; Dragesco, 1965).

The original descriptions of *Tracheloraphis striatus* Raikov, 1962 and *T. remanei* Dragesco, 1960 are based solely on stained specimens, and no information on their live morphology is available. Nevertheless, both can be separated from *T. katzae* spec. nov. by having a narrower glabrous stripe (about double distance between two adjacent somatic kineties, and one-third of the body width, respectively vs. about as wide as the body) (Figures 8D,E; Dragesco, 1960; Raikov, 1962).

Although no information on the infraciliature of *Trachelocerca schulzei* Dragesco, 1960 is available, based on

the original illustration, it can be separated from *T. katzae* spec. nov. by its larger ratio of body length to body width (about 35:1 vs. about 20:1) in fully extended cells (Figure 8F; Dragesco, 1960).

Tracheloraphis colubis (Kahl, 1933) Xu et al., 2011; Figures 9, 10 and Table 3)

This species was originally reported by Kahl (1933) and redescribed in detail by Xu et al. (2011a). Our population matches both descriptions very well, therefore redescription and an improved diagnosis based on the present and previous populations are provided here.

Improved Diagnosis

Extended cells 250–1,000 μm \times 20–50 μm *in vivo*; claviform tail. Seventeen to thirty-one somatic kineties. Glabrous stripe narrow, width about equal to gap between two adjacent somatic kineties. Four macronuclei in a single group. Cortical granules circular in outline when viewed from above, elliptical in lateral view 1.5–2 μm \times 2.5 μm , colorless.

Redescription Based on Qingdao Population

Extended cells 250–600 μm \times 30–50 μm *in vivo*; body flattened about 3:1, flexible and contractile (Figures 9A–C, 10A–C); neck and tail indistinctly separated from trunk, head triangular and conspicuous, tail claviform (Figures 9A–C, 10A–C). Cortical granules ellipsoidal, 1.5–2 μm \times 2.5 μm , colorless in bright field

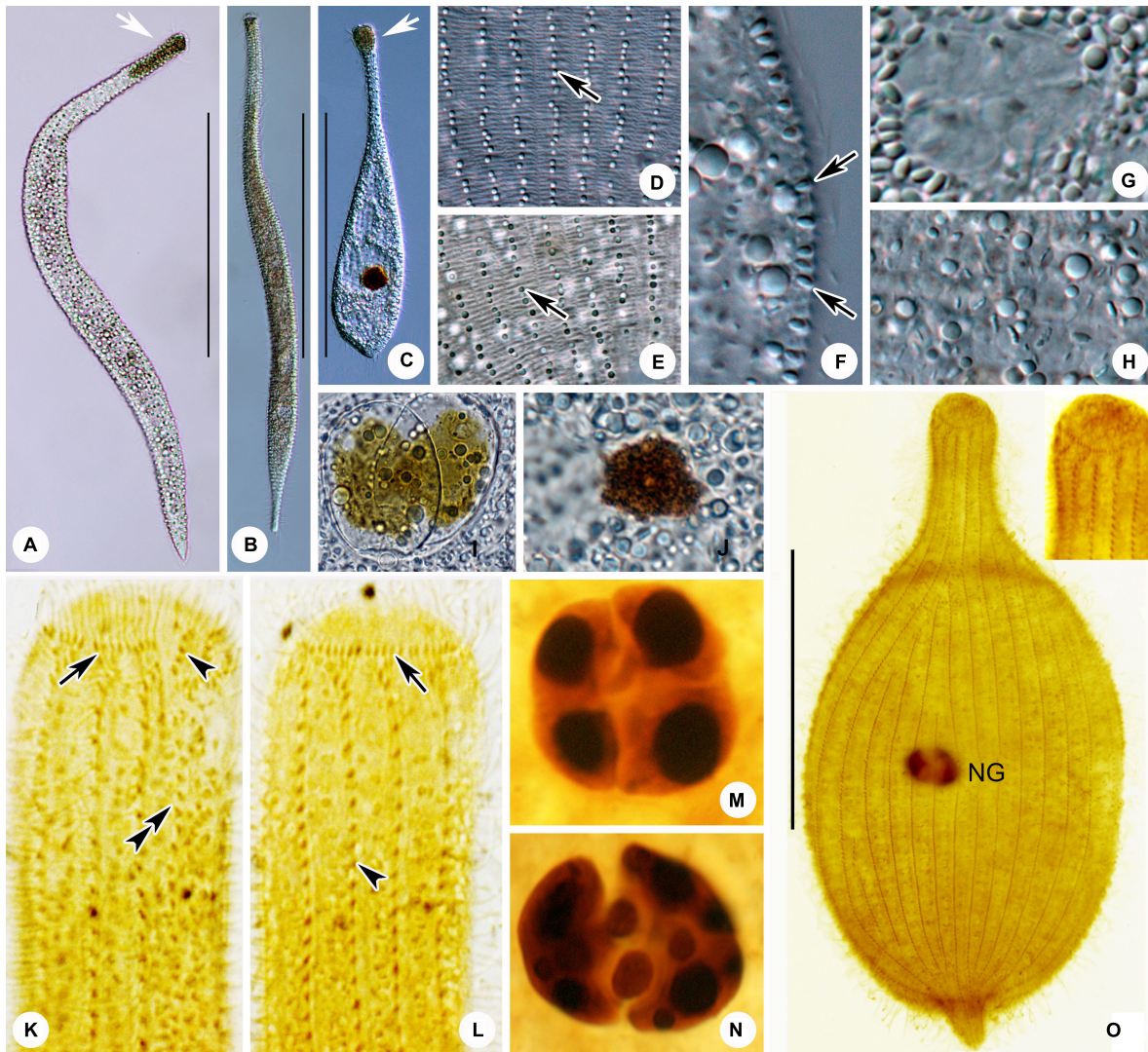


FIGURE 10 | (A–O) *Tracheloraphis colubis* *in vivo* (A–J) and after protargol staining (K–O). (A,B) Typical individuals. Arrowhead showing the head of cell. (C) An extremely contracted cell. Arrowhead showing the head of cell. (D,E) Distribution of cortical granules between somatic kineties (arrow). (F) Lateral view, showing the elliptical cortical granules at the cell margin (arrows). (G) Nuclear group. (H) Showing granules in cell. (I,J) Showing algae in the food vacuole. (K,L) Detailed ciliature of left (K) and right (L) side of anterior body region. Arrow in (K) showing circummoral kinety, arrowhead showing brosse, double arrowheads showing bristle kinety. Arrow in (L) showing circummoral kinety, arrowhead showing somatic kinety. (M,N) Nuclear group. (O) Overview showing ciliary pattern. NG, nuclear group. Scale bars = 300 μm (A–C); 100 μm (O).

at high magnification; round when viewed from above, elliptical in lateral view; arranged in rows between somatic kineties and sparsely distributed in glabrous stripe (Figures 9E,F, 10D–F). Cytoplasm colorless and transparent, packed with cytoplasmic granules, ellipsoidal, 1–3 μm long and colorless (Figure 10H). Some food vacuoles containing algae (Figures 9C, 10C,I,J).

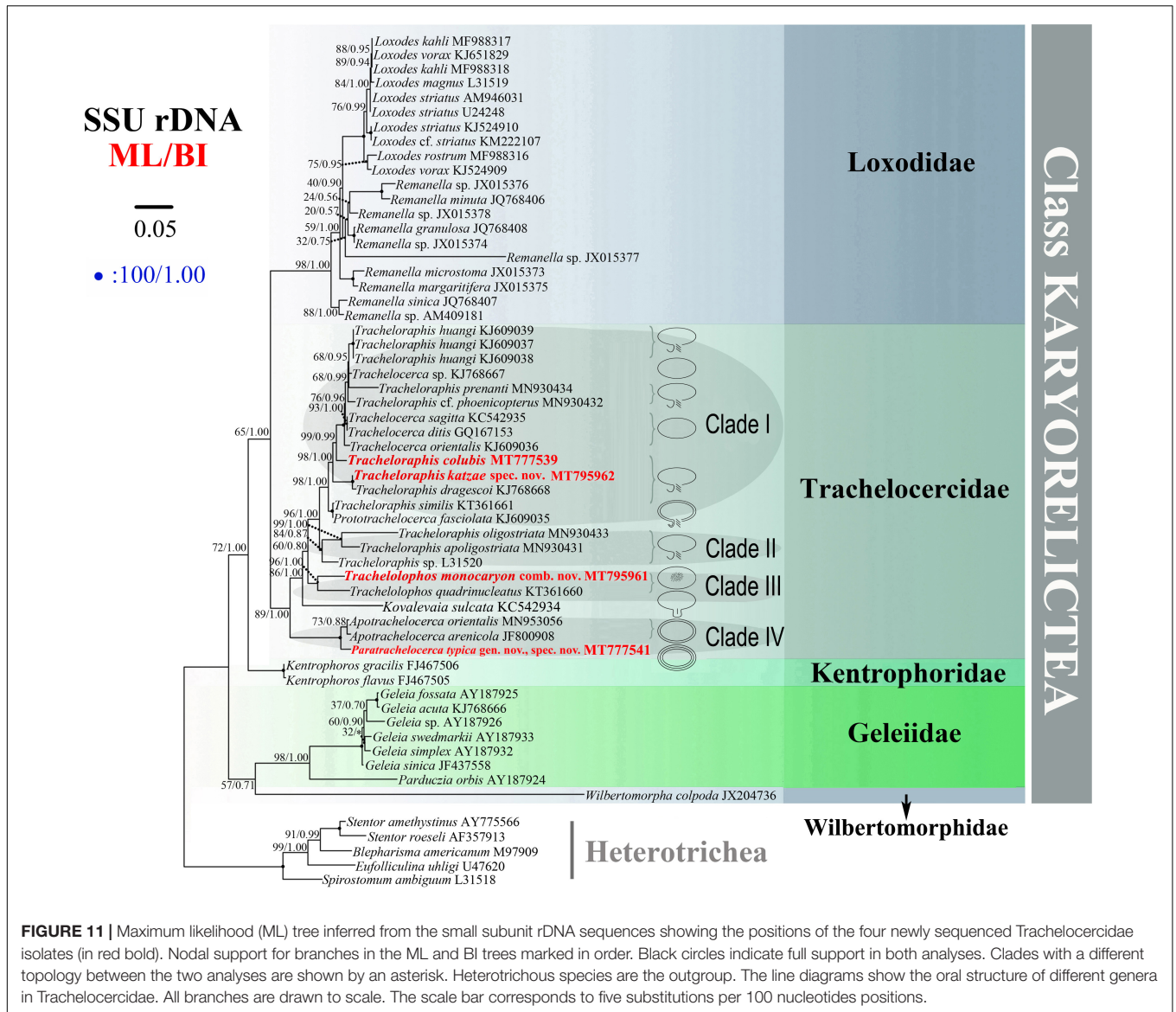
Locomotion by gliding, winding between sand grains and organic debris.

Entire infraciliature consisting of dikinetids (Figures 9I, 10O). Somatic cilia about 10 μm long *in vivo* and arranged in longitudinal rows. Usually one brosse kinety (Figures 9G,H, 10K,L). Glabrous stripe very narrow, width about equal to gap between two adjacent somatic kineties, and bordered by

irregularly spaced bristle kinety (Figures 9G, 10K). Seventeen to 27 somatic kineties. Anterior and posterior of secant system formed on left side of glabrous stripe, some kineties abut to bristle kinety (Figures 9G, 10K). Four macronuclei and two micronuclei in a single group (Figures 9D, 10G,M,N).

Discussion

There have been several redescrptions of *Tracheloraphis colubis* since it was first reported (Kahl, 1933; Raikov, 1963; Xu et al., 2011a). Kahl (1933) and Raikov (1963) both assigned this species to the genus *Trachelocerca* due to its curved posterior end and narrow glabrous stripe. However, the shape and structure of the oral ciliature are the most important



characters for generic classification (Foissner and Dragesco, 1996a,b; Foissner and Al-Rasheid, 1999). Therefore, Xu et al. (2011a) assigned this species to the genus *Tracheloraphis*. The present population matches the population described by Xu et al. (2011a), so we have no doubt that these two populations are conspecific.

Molecular Phylogeny Based on SSU rDNA Sequence Data (Figure 11)

The length (bp), GC content and Genbank accession numbers of the new SSU rDNA sequences of the four isolates are as follows: *Paratrachelocerca typica* gen. nov., spec. nov.—1,557, 48.62%, MT777541; *Trachelolophos monocaryon* (Dragesco, 1965) comb. nov.—1,473, 48.47%, MT795961; *Tracheloraphis katzae* spec. nov.—1,520, 47.89%, MT795962; *Tracheloraphis colubis*—1,597, 47.65%, MT777539.

The ML and BI trees have similar topologies, therefore only the ML tree is presented (Figure 11). The family Trachelocercidae is a well-supported monophyletic group (89% ML, 1.00 BI) that is sister to the family Loxodidae (65% ML, 1.00 BI). Within Trachelocercidae there are four main clades (Clades I, II, III, and IV). *Tracheloraphis colubis* is sister to several *Tracheloraphis* and *Trachelocerca* species. *Tracheloraphis katzae* spec. nov. groups with *T. dragescoi* within Clade I. *Prototrachelocerca fasciolata* shows a close relationship with *Tracheloraphis similis* which together occupy the basal position within Clade I. *Tracheloraphis* species interdigitate with those of *Trachelocerca* resulting in the non-monophyly of both genera, which is consistent with previous studies (Xu et al., 2014; Yan et al., 2016). Clade II consists of three *Tracheloraphis* species. Two *Trachelolophos* species group together forming a sister branch to Clades I and II. Clade III comprises *Trachelolophos monocaryon* and *T. quadrinucleatus*. *Kovalevaia sulcata* is sister group to the assemblage of Clades

I–III. Clade IV comprises *Paratrachelocerca typica* and two species of *Apotrachelocerca*.

Based on the combination of morphological features and molecular evidence, Yan et al. (2016) suggested that *Apotrachelocerca*, which possesses two uninterrupted rows of circumoral kineties, is the closest relative to the common ancestor of trachelocercids, followed by *Kovalevaia*, *Trachelolophos* and *Prototrachelocerca* and that *Trachelocerca*, *Tracheloraphis* and possibly *Sultanophrys* derived from *Prototrachelocerca*. Our results mainly support this hypothesis except that *Kovalevaia* forms a sister branch with Clades I–III (of which Clade III comprises two *Trachelolophos* species) rather than grouping directly with *Trachelolophos* (Figure 11). In addition, the early branching of *Paratrachelocerca* gen. nov. within Clade IV provides further evidence that multiple rows of uninterrupted circumoral kineties is probably an ancestral feature.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the online repositories. The names of the repository/repositories and accession number(s) are as follows: <https://www.ncbi.nlm.nih.gov/genbank/>, MT777541; <http://zoobank.org/>, urn:lsid:zoobank.org:pub:913C263C-56A7-40AC-9885-E06DC530A6D8; <https://www.ncbi.nlm.nih.gov/genbank/>, MT795961; <https://www.ncbi.nlm.nih.gov/genbank/>, MT795962; <https://www.ncbi.nlm.nih.gov/genbank/>, MT777539.

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AUTHOR CONTRIBUTIONS

YY and YW conceived to the study. MM, YL, and YY carried out the live observation, protargol staining, DNA extraction, and data analyses. All authors contributed to the writing of the manuscript. YY, MM, and AW contributed to the revision and all authors approved the final 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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