



Morphology and phylogeny of three trachelocercids (Protozoa, Ciliophora, Karyorelictea), with description of two new species and insight into the evolution of the family Trachelocercidae

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Although trachelocercid ciliates are common in marine sandy intertidal zones, methodological difficulties mean that their biodiversity and evolutionary relationships have not been well documented. This paper investigates the morphology and infraciliature of two novel *Trachelolophos* and one rarely known form, *Tracheloraphis similis* Raikov and Kovaleva, 1968, collected from the coastal waters of southern and eastern China. The small subunit (SSU) rRNA gene sequences of two of the species are presented, allowing the phylogenetic position of the genus *Trachelolophos* to be revealed for the first time. Phylogenetic analyses based on SSU rRNA gene sequences indicate that *Trachelolophos* branches with *Kovalevaia* and forms a sister clade with the group including *Prototrachelocerca*, *Trachelocerca* and *Tracheloraphis*. The monophyly of *Trachelocerca* is not rejected by the approximately unbiased (AU) test ($P = 0.209$, > 0.05) but that of *Tracheloraphis* is rejected ($P = 3e-033$, < 0.05). The newly sequenced genus *Trachelolophos*, and recent studies on the morphology and phylogeny of the family Trachelocercidae, suggest two new hypotheses about the evolution of the seven genera within Trachelocercidae, based on either infraciliature or molecular evidence. Both hypotheses suppose the compound circumoral kineties in the oral apparatus is a plesiomorphic feature while the single circumoral kinety is synapomorphic. More evidence is still needed, however, as to whether the closed circumoral kinety with no brosse feature in *Trachelocerca* is ancestral or secondarily reduced.

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ADDITIONAL KEYWORDS: evolution – new species – phylogeny – SSU rRNA gene – Trachelocercidae.

INTRODUCTION

Trachelocercid ciliates are common in marine sandy intertidal zones (Borror, 1968; Fenchel, 1969; Patterson, Larsen & Corliss, 1989; Carey, 1992; Al-Rasheid, 1996, 1997, 1998, 2001; Foissner & Dragesco, 1996a; Al-Rasheid & Foissner, 1999). To date, more than 70 trachelocercids belonging to seven genera have been

reported in marine benthic environments, and 22 small subunit (SSU) rRNA gene sequences, including seven environmental sequences, are available in GenBank (Hirt *et al.*, 1995; Andreoli *et al.*, 2009; Mazei *et al.*, 2009; Gao *et al.*, 2010; Xu *et al.*, 2011a,b, 2014; Yan *et al.*, 2013, 2015). Since the new standards for classification of genera were established, and the infraciliature of more species was revealed (Foissner, 1996; Foissner & Dragesco, 1996a,b), the oral ciliature has acquired greater importance in generic identification and evolutionary reconstruction. Thus far, seven genera have

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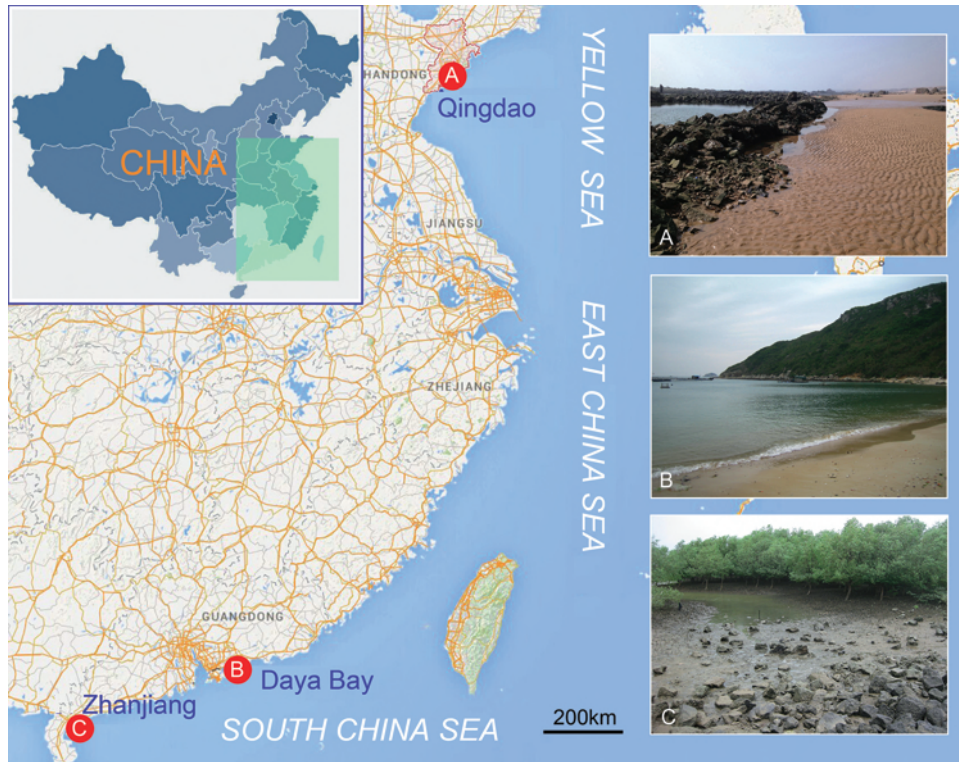


Figure 1. Photographs of the sample site. A, an intertidal zone of a sandy beach in Qingdao ($35^{\circ}55'45''\text{N}$, $120^{\circ}12'59''\text{E}$); B, an intertidal zone of a sandy beach in Daya Bay, Guangzhou ($22^{\circ}46'32''\text{N}$, $114^{\circ}40'13''\text{E}$); C, a mangrove wetland in Zhanjiang ($21^{\circ}09'40''\text{N}$, $110^{\circ}25'38''\text{E}$).

been reported within this family, with six explicitly different kinds of oral structure (Xu *et al.*, 2011a). Only *Sultanophrys* and *Tracheloraphis* share the same oral ciliature (Foissner & Al-Rasheid, 1999).

Although the evolution within trachelocercids is difficult to follow for many reasons, such as the homogeneity of the somatic infraciliature (Foissner & Dragesco, 1996b), previous studies have offered some suggestions (Foissner, 1996, 1997, 1998; Foissner & Dragesco, 1996b): (1) *Prototrachelocerca* has been considered to be separated from other trachelocercids at the family level because of its compound circumoral ciliature, which somewhat resembles the oral structures in loxodids and is therefore thought to represent an ancestral pattern; (2) the keyhole-shaped circumoral kinety in *Kovalevaia* probably represents a particular evolutionary branch; and (3) *Trachelocerca* is difficult to place, and its closed simple circumoral kinety might be a secondarily reduced feature, which would make it the most evolved member within the family. The lack of molecular information available at the time these studies were made means, however, that all these hypotheses are based purely on morphology. This situation has now changed, with, currently, 22 SSU rRNA gene sequences of trachelocercids available in GenBank, including nine environmental se-

quences. With *Trachelolophos* newly sequenced in this paper, six of the seven genera within Trachelocercidae now have molecular information available. This combination of morphological and phylogenetic data on trachelocercids (Xu *et al.*, 2011a, 2014; Yan *et al.*, 2013, 2015) provides an opportunity for a better analysis of the evolutionary relationships within this family.

In the present paper, we therefore describe two novel *Trachelolophos* and one poorly known *Tracheloraphis* species that were isolated from the coastal waters of southern and eastern China (Fig. 1). All these species were investigated both *in vivo* and using the protargol staining method, and the molecular phylogeny of two of them was analysed based on SSU rRNA gene sequence data. These data enable the phylogenetic position of the genus *Trachelolophos* to be revealed for the first time, and we use the new data to suggest two evolutionary hypotheses for the trachelocercids.

MATERIAL AND METHODS

COLLECTION AND IDENTIFICATION

Trachelolophos quadrinucleatus sp. nov. was collected on 23 November 2012 from a mangrove wetland on Techeng island, Zhanjiang, China ($21^{\circ}09'40''\text{N}$,

110°25'38"E), where the water temperature was 26 °C and salinity about 25‰ (Fig. 1C). *Trachelolophos binucleatus* sp. nov. was sampled on 24 May 2013 from the intertidal zone of a bathing beach in Qingdao, China (35°55'45"N, 120°12'59"E), where the water temperature was 16 °C and salinity about 33‰ (Fig. 1A). *Tracheloraphis similis* was collected on 13 December 2012 from the intertidal zone of Daya Bay, Guangzhou, China (22°46'32"N, 114°40'13"E), where the water temperature was 22 °C and salinity about 32‰ (Fig. 1B). Sampling methods largely followed those of Fan *et al.* (2014). Briefly, sand (the top 5 cm), or sediment with seawater, was taken from the site. Cells were isolated and observed *in vivo* using an oil immersion objective and differential interference microscopy (Xu *et al.*, 2015). The infraciliature was revealed by using the protargol staining method (Wilbert, 1975). Counts and measurements on impregnated specimens were conducted at magnifications ranging between ×100 and ×1000. Drawings were based on free-hand sketches or with the help of a camera lucida. Terminology and systematics are according to Foissner & Dragesco (1996a) and Lynn (2008), respectively.

DNA EXTRACTION, GENE SEQUENCING AND PHYLOGENETIC ANALYSES

DNA extraction was performed using the DNeasy Tissue Kit (Qiagen) according to Chen *et al.* (2015). The primers used for SSU rRNA gene amplification was a forward primer (5'-GCCAGTAGTSATATGCTTGTCT-3') designed by our colleague, Mr Weibo Zheng (unpublished) and a universal eukaryotic reverse primer (5'-TGATCCTTCTGCAGGTTACACTAC-3') (Elwood, Olsen & Sogin, 1985; Medlin *et al.*, 1988). PCR amplification and sequencing of the SSU rRNA gene were performed according to the method given by Xu *et al.* (2013).

Additional sequences were downloaded from the GenBank database (for accession numbers, see Fig. 8). Alignment of the SSU rRNA gene sequences was conducted using the GUIDANCE algorithm (Penn *et al.*, 2010a) following the default parameters in the GUIDANCE web server (Penn *et al.*, 2010b). Ambiguous columns in the alignment were defined as those which fell below a confidence score of 0.696, as calculated by GUIDANCE, and were removed. The resulting curated alignment included 1674 characters of 51 taxa. *Spirostomum ambiguum*, *Eufolliculina uhligi*, *Blepharisma americanum*, *Stentor amethystinus* and *S. roeseli* were used as outgroup taxa. Bayesian inference (BI) analysis was performed with MrBayes 3.2.3 on XSEDE on the CIPRES Science Gateway v.3.3 (<http://www.phylo.org>; Miller, Pfeiffer & Schwartz, 2010) using the GTR+I+G model. The chain length of the Bayesian analysis was 1000 000 generations with sampling every 100 generations. The first 25% of the

sampled trees were considered as burn-in. Maximum-likelihood (ML) analysis was carried out with 1000 replicates online on the CIPRES Science Gateway v.3.3 (<http://www.phylo.org>; Miller *et al.*, 2010) using RAxML-HPC2 on XSEDE with the GTR+I+G model (Stamatakis, Hoover & Rougemont, 2008).

The statistical possibility of the alternative phylogenetic hypotheses was evaluated using approximately unbiased (AU) tests (Shimodaira, 2002). Constrained ML trees compelling the monophyly of *Trachelocerca*, *Tracheloraphis*, *Tracheloraphis* without *Tracheloraphis* sp. (L31520), and then, in turn, without *Trachelocerca* and *Tracheloraphis*, were generated using the same toolkit as for the unconstrained ML trees. The resulting constrained topologies were then compared with the non-constrained ML topologies using the AU test option implemented in CONSEL v.0.1i (Shimodaira & Hasegawa, 2001).

RESULTS AND DISCUSSION

CLASS KARYORELICTEA CORLISS, 1974
FAMILY TRACHELOCERCIDAE KENT, 1881
GENUS *TRACHELOLOPHOS* FOISSNER & DRAGESCO, 1996

TRACHELOLOPHOS QUADRINUCLEATUS SP. NOV. (FIGS 2, 3; TABLE 1)

Diagnosis: Body size *in vivo* 1100–1400 × 25–40 µm; 14–25 and 26–40 somatic kineties on head and trunk, respectively; single nuclear group composed of three or four macronuclei and two micronuclei; glabrous stripe narrow, corresponding to area occupied by two somatic kineties; cortical granules colourless and about 0.5 µm in diameter.

Type locality: A mangrove wetland on Techeng island, Zhanjiang, China (21°09'40"N, 110°25'38"E), where the water temperature was 26 °C and salinity about 25‰ (Fig. 1C).

Type specimens: A protargol-impregnated slide containing the holotype specimen marked with an ink circle is deposited in the Laboratory of Protozoology, Ocean University of China, China (No. YY2012112308). One paratype slide is deposited in the Natural History Museum, London, UK, with registration number NHMUK 2015.9.15.1.

Etymology: The species-group name *quadrinucleatus* reflects the fact that this organism usually possesses four macronuclei.

Description: Fully extended cells about 1300 × 35 µm *in vivo*; body flexible and contractile (Figs 2A–C, 3A–D). Cell distinctly tripartite, with neck, tail and trunk

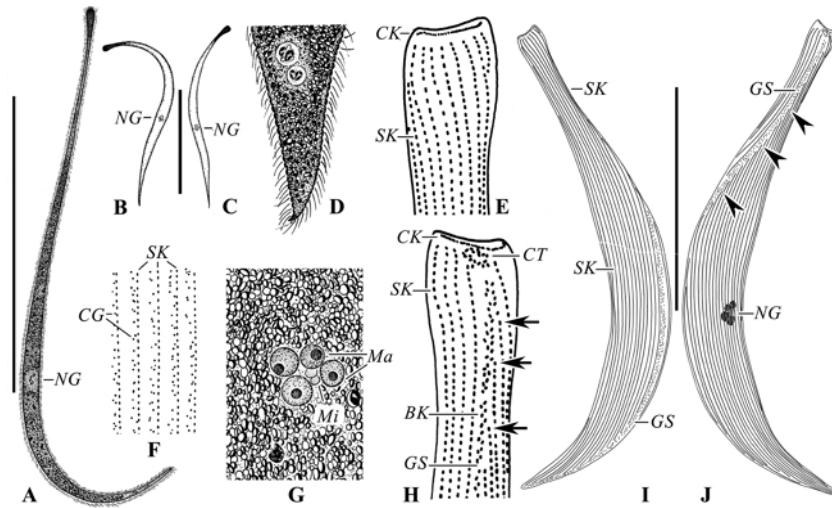


Figure 2. *Trachelolophos quadrinucleatus* sp. nov. from life (A–D, F, G) and after protargol staining (E, H–J). A, typical individual; B, C, different body shapes; D, wedge-like tail; E, H, infraciliature of anterior end, showing circumoral kinety, ciliary tuft, narrow glabrous stripe and bristle kinety; arrows point to anterior secant system forming on the left side of glabrous stripe; F, cortical granules arranged in line between somatic kineties; G, four macronuclei and two micronuclei forming a nuclear group; I, J, general infraciliature of the holotype specimen, marking single nuclear group, narrow glabrous stripe and anterior secant system forming on the left side of glabrous stripe (arrowheads). Abbreviations: BK, bristle kinety; CG, cortical granules; CK, circumoral kinety; CT, ciliary tuft; GS, glabrous stripe; Ma, macronuclei; Mi, micronuclei; NG, nuclear group; SK, somatic kineties. Scale bars: 500 μ m in A–C; 200 μ m in I, J.

regions (Figs 2A–C, 3A–C). Head conspicuously claviform; tail wedge-shaped (Figs 2A, D, 3A, F). Body colour dark brown at low magnification due to multiple refractile inclusions, with several food vacuoles containing ingested algae (Figs 2D, G, 3E, F). Single nuclear group located in centre of trunk, containing three or four macronuclei, 7–10 μ m in diameter, and two micronuclei, 2–4 μ m in diameter (Figs 2G, 3E, H, I). Colourless cortical granules, c. 0.5 μ m in diameter, scattered between ciliary rows, which are not found in glabrous stripe (Figs 2F, 3G). Locomotion by gliding between sand grains and organic debris.

Cell surface densely ciliated with unciliated zone, glabrous stripe, about as wide as two somatic kineties (Figs 2I, J, 3J–M). Entire infraciliature consisting of dikinetids. About 18 and 35 somatic kineties on head and trunk, respectively, with cilia c. 13 μ m long. Anterior and posterior secant system formed on left side of glabrous stripe where some kineties abut to bristle kinety (Figs 2H, J, 3K, M). Oral infraciliature consisting of uninterrupted circumoral kinety with cilia about 5 μ m long and roundish patch of disordered dikinetids in centre of oral cavity with cilia forming conspicuous tuft (Figs 2E, H, 3K, L).

Comparison: Since the genus *Trachelolophos* was established by Foissner & Dragesco (1996b), only the following two species have been reported.

Trachelolophos filum (Dragesco & Dragesco-Kernéis, 1986) resembles the new species in the number of somatic kineties on trunk. Although there is no *in vivo* information available for this species, it clearly differs from our new species in possessing more macronuclei (6–30 forming a strand vs. three or four macronuclei forming a single nuclear group) (Foissner & Dragesco, 1996b).

Trachelolophos gigas Foissner & Dragesco, 1996 has a similar body shape to the new form, but can be distinguished from the latter by having a larger size (2000 μ m vs. 1100–1400 μ m), a conspicuously higher number of somatic kineties on the trunk (52–71 vs. 26–40) and many more macronuclei (17–33 macronuclei forming a strand vs. three or four macronuclei forming a nuclear group) (Foissner & Dragesco, 1996b).

***TRACHELOLOPHOS BINUCLEATUS* SP. NOV.**

(FIGS 4, 5; TABLE 1)

Diagnosis: Body size *in vivo* 500–1000 \times 25–35 μ m; 9–19 and 17–26 somatic kineties on head and trunk, respectively; single nuclear group composed of two or three macronuclei and one micronucleus; narrow glabrous stripe, corresponding to area occupied by two somatic kineties; cortical granules colourless and about 0.5 μ m in diameter.

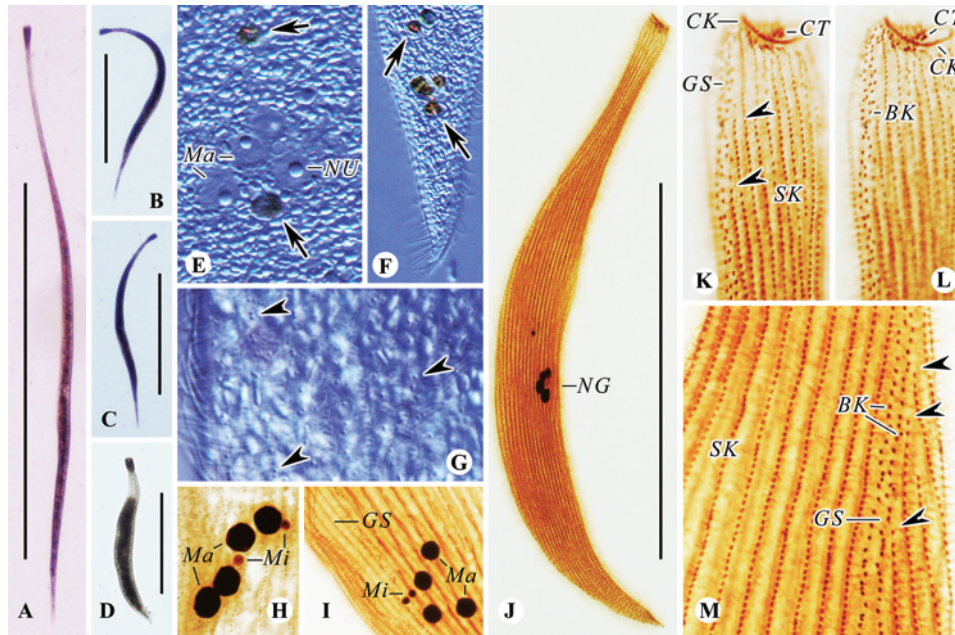


Figure 3. *Trachelolophos quadrinucleatus* sp. nov. from life (A–G) and after protargol staining (H–M). A, typical individual; B, C, different body shapes; D, contracted cell; E, macronuclei forming a nuclear group and food vacuoles (arrows); F, wedge-shaped tail, noting food vacuoles including ingested algae (arrows); G, small colourless cortical granules arranged in line between somatic kineties (arrowheads); H, I, four macronuclei and two micronuclei forming a nuclear group; J, general infraciliature, marking single nuclear group; K, L, infraciliature of anterior end, showing circumoral kinety, ciliary tuft, narrow glabrous stripe and bristle kinety; arrowheads point to anterior secant system forming on the left side of glabrous stripe; M, infraciliature of anterior portion indicating narrow glabrous stripe, bristle kinety and anterior secant system (arrowheads). Abbreviations: BK, bristle kinety; CK, circumoral kinety; CT, ciliary tuft; GS, glabrous stripe; Ma, macronuclei; Mi, micronuclei; NG, nuclear group; NU, nucleolus; SK, somatic kineties. Scale bars: 500 μ m in A–D; 200 μ m in J.

Type locality: The intertidal zone of a bathing beach in Qingdao, China (35°55'45"N, 120°12'59"E), where the water temperature was 16 °C and salinity about 33‰ (Fig. 1A).

Type specimens: A protargol-impregnated slide containing the holotype specimen marked with an ink circle is deposited in the Laboratory of Protozoology, Ocean University of China, China (No. YY2013052403). One paratype slide is deposited in the Natural History Museum, London, UK, with registration number NHMUK 2015.9.15.2.

Etymology: The species-group name *binucleatus* reflects the fact that this organism usually has two macronuclei.

Description: Fully extended cells about 700 \times 30 μ m *in vivo*; body flexible and flattened ribbon-like with claviform head and pointed tail (Figs 4A–C, 5A–C). Body colour dark at low magnification with neck and tail portion transparent due to packed inclusions

(Figs 4A, D, 5A, D). Single nuclear group located in centre of trunk, containing two or three macronuclei, 7–10 μ m in diameter, and one micronucleus, 3–6 μ m in diameter (Figs 4A, D, I, 5D, G, H). Colourless cortical granules, c. 0.5 μ m in diameter, arranged in line between ciliary rows and scattered in glabrous stripe (Figs 4F, 5E, F). Locomotion by gliding between sand grains and organic debris.

Cell surface densely ciliated with unciliated zone, glabrous stripe, about as wide as two somatic kineties (Figs 4F, H, 5J, M). Entire infraciliature consisting of dikinetids with cilia c. 10 μ m long (Figs 4H, I, 5G). About 14 and 19 somatic kineties on head and trunk, respectively. Anterior and posterior secant system formed on left side of glabrous stripe where some kineties abut to bristle kinety (Figs 4F, H, 5J, M). Oral infraciliature consisting of uninterrupted circumoral kinety and conspicuous ciliary tuft located in centre of oral cavity (Figs 4F, G, 3I, L).

Comparison: Similar to *Trachelolophos quadrinucleatus* sp. nov., the current new species should be compared with its known congeners.

Table 1. Morphometric characteristics of *Trachelolophos quadrinucleatus* sp. nov. (upper line), *Trachelolophos binucleatus* sp. nov. (middle line) and *Tracheloraphis similis* (lower line) from protargol-impregnated specimens

Character	Min.	Max.	Median	Mean	SD	CV	N
Body length	254	574	374	407.3	111.3	27.3	7
	263	598	368	395.6	107.7	27.2	17
	199	622	283	322.5	102.9	31.9	25
Body width	46	100	70	72.9	16.5	22.7	7
	24	64	45	46.0	9.6	20.9	17
	74	141	100	102.3	19.2	18.8	24
NG, number	1	1	1	1.0	0	0	15
	1	1	1	1.0	0	0	12
	2	2	2	2.0	0	0	24
Ma, number	3	4	4	3.8	0.4	10.9	15
	2	3	2	2.1	0.3	13.9	12
	6	11	8	7.9	0.9	11.5	26
Mi, number	2	2	2	2.0	0	0	15
	1	1	1	1.0	0	0	12
	2	2	2	2.0	0	0	26
Ma in 1 st NG, number	–	–	–	–	–	–	–
	–	–	–	–	–	–	–
	2	5	4	3.8	0.6	15.9	26
Ma in 2 nd NG, number	–	–	–	–	–	–	–
	–	–	–	–	–	–	–
	3	6	4	4.0	0.4	11.0	26
SK, head	14	25	16	17.7	3.9	22.3	7
	9	19	11	14.0	2.6	22.4	14
	13	16	15	14.9	0.8	5.4	21
SK, trunk	26	40	36	34.6	4.8	13.8	7
	17	26	18	19.3	2.3	11.8	14
	18	21	19	19.0	0.9	4.7	22
Anterior end to NG	173	291	248	274.1	42.4	17.2	7
	145	354	216	226.9	66.4	29.2	16
	76	183	111	117.7	27.7	23.5	26

All measurements in μm . Abbreviations: CV, coefficient of variation (%); Ma, macronuclei; Mi, micronuclei; NG, nuclear groups; SD, standard deviation; SK, somatic kineties; N, number of specimens.

Trachelolophos filum can be separated from the new species by having more somatic kineties on the trunk (26–35 vs. 17–26) and more macronuclei (6–30 forming a strand vs. two or three forming a nuclear group) (Foissner & Dragesco, 1996b).

Trachelolophos gigas differs from *T. binucleatus* sp. nov. in possessing a longer body length (2000 μm vs. 500–1000 μm), more somatic kineties on the trunk (52–71 vs. 17–26) and more macronuclei (17–33 macronuclei forming a strand vs. two or three forming a nuclear group) (Foissner & Dragesco, 1996b).

Trachelolophos binucleatus sp. nov. differs from *T. quadrinucleatus* sp. nov. (above) in having far fewer somatic kineties on the trunk (17–26 vs. 26–40) and fewer macronuclei (two or three vs. three or four).

GENUS *TRACHELORAPHIS* DRAGESCO, 1960

TRACHELORAPHIS SIMILIS RAIKOV & KOVALEVA, 1968 (FIGS 6, 7; TABLE 1)

This species was reported by Raikov & Kovaleva (1968) and no redescrptions have been made since then. Furthermore, in the original report it was insufficiently described and no details about the live morphology, or drawings, are available. Hence, an improved definition and a redescription, based mainly on the Chinese population, are presented here.

Improved diagnosis: Body size *in vivo* 800–1500 \times 20–40 μm ; 13–16 and 18–21 somatic kineties on head and trunk, respectively; two nuclear groups, each of which is composed of about four macronuclei and two

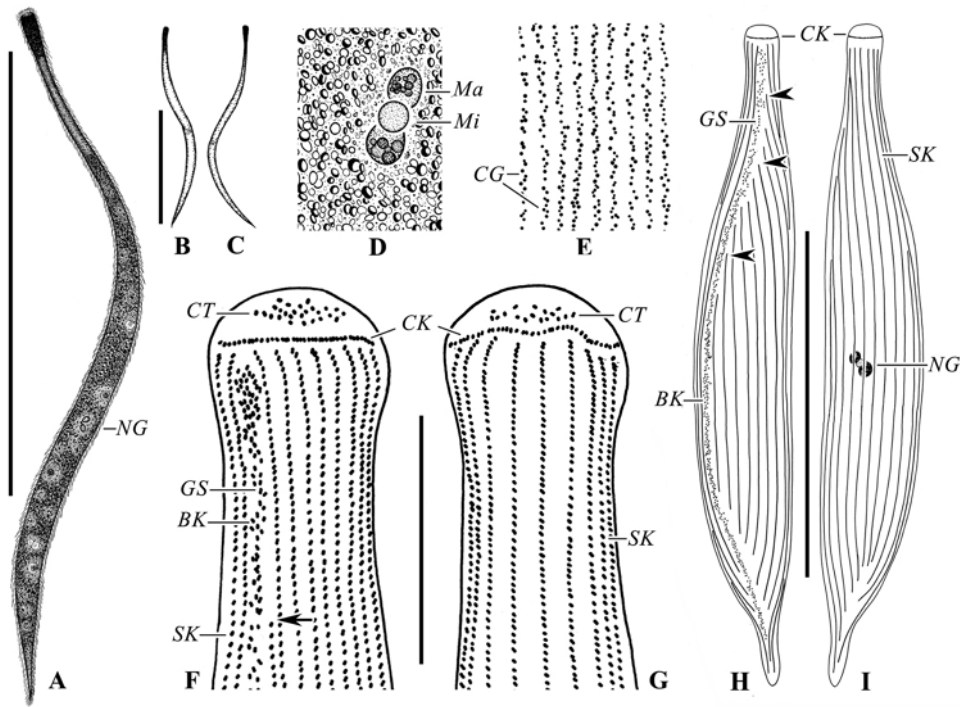


Figure 4. *Trachelolophos binucleatus* sp. nov. from life (A–E) and after protargol staining (F–I). A, typical individual; B, C, different body shapes; D, two macronuclei and one micronucleus forming a nuclear group; E, small cortical granules arranged in line between somatic kineties; F, G, infraciliature of anterior end, indicating circumoral kinety, ciliary tuft, narrow glabrous stripe and bristle kinety; arrow in F shows anterior secant system on the left side of glabrous stripe; H, I, general infraciliature of the holotype specimen, to show single nuclear group, narrow glabrous stripe, bristle kinety and anterior secant system (arrowheads). Abbreviations: BK, bristle kinety; CG, cortical granules; CK, circumoral kinety; CT, ciliary tuft; GS, glabrous stripe; Ma, macronuclei; Mi, micronucleus; NG, nuclear group; SK, somatic kineties. Scale bars: 400 μm in A–C; 30 μm in F, G; 200 μm in H, I.

micronuclei; glabrous stripe corresponding to area occupied by five or six somatic kineties; cortical granules yellowish and *c.* 0.5 μm in diameter.

Deposition of voucher material: A voucher slide with protargol-impregnated specimens is deposited in the Laboratory of Protozoology, Ocean University of China (No. YY2012121301).

Redescription based on the Chinese population: Fully extended cells about $1200 \times 30 \mu\text{m}$ *in vivo*; body flexible and contractile with cross-section elliptical; cell distinctly tripartite, with neck, tail and trunk regions (Figs 6A, 7A). Head conspicuously claviform; tail wedge-shaped (Figs 6A, B, 7A, C). Endoplasm greyish and opaque due to multiple refractile inclusions (Figs 6B, C, 7B–D). Two nuclear groups, each of which contains about four macronuclei, 9–15 μm in diameter, and two micronuclei, *c.* 4–5 μm in diameter (Figs 6A, C, H, 7B, G, I, J). Small yellowish cortical granules, *c.* 0.5 μm in diameter, distributed between ciliary rows and in

glabrous stripe (Figs 6D, 7E, F). Locomotion by gliding between sand grains and organic debris.

Cell surface densely ciliated with unciliated zone, glabrous stripe, about as wide as five or six somatic kineties (Figs 6E, G, H, 7G, H, L). Entire infraciliature consisting of dikinetids with cilia about 10 μm long (Figs 6G, H, 7G). About 15 and 19 somatic kineties on head and trunk, respectively. Anterior and posterior secant system formed on left side of glabrous stripe where some kineties abut to bristle kinety (Figs 6F, H, 7L). Oral ciliature consisting of circumoral kinety, which is interrupted by three inserted brosse kineties (Figs 6E–H, 7H, L).

Remarks: Based on the original description given by Raikov & Kovaleva (1968), this species has an elongated spindle-shaped body, a pointed tail that forms a slight hook and a glabrous stripe as wide as six kineties. Given these characteristics, this Guangzhou population corresponds well to the original report. Raikov & Kovaleva (1968) describe *Tracheloraphis similis* as colourless but provide no information on its

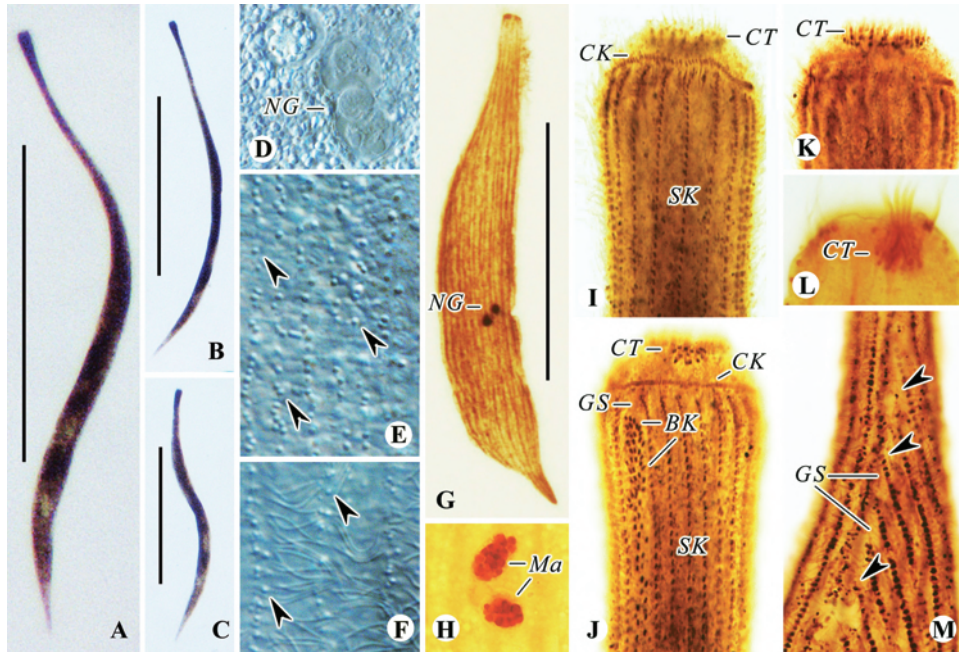


Figure 5. *Trachelolophos binucleatus* sp. nov. from life (A–F) and after protargol staining (G–M). A, typical individual; B, different body shape; C, slightly contracted cell; D, single nuclear group; E, F, small colourless cortical granules arranged in line between somatic kineties (arrowheads); G, general infraciliature, to show single nuclear group; H, two macronuclei forming a nuclear group; I–L, infraciliature of anterior end, indicating circumoral kinety, ciliary tuft, narrow glabrous stripe and bristle kinety; M, infraciliature of anterior portion, noting narrow glabrous stripe and anterior secant system forming on the left side of it (arrowheads). Abbreviations: BK, bristle kinety; CK, circumoral kinety; CT, ciliary tuft; GS, glabrous stripe; Ma, macronuclei; NG, nuclear group; SK, somatic kineties. Scale bars: 400 μ m in A–C; 200 μ m in G.

cortical granules. Based on our study, however, this species has yellowish cortical granules but they are so small (c. 0.5 μ m in diameter) that the whole cell looks colourless at low magnification. The other minor differences between these two populations are the body length *in vivo* (600–800 μ m vs. 800–1500 μ m in Guangzhou population) and the number of somatic kineties (16 vs. 13–16 on head, 18–21 on trunk in Guangzhou population). These differences are probably population-dependent because these values overlap with each other. Consequently, we identified the Guangzhou isolate as a population of *T. similis*.

MOLECULAR PHYLOGENY BASED ON SSU rRNA GENE SEQUENCES

The length (bp), GC content and GenBank accession numbers of the two species are as follows: *Trachelolophos quadrinucleatus* sp. nov. – 1554, 47.04%, KT361660; *Tracheloraphis similis* – 1628, 47.91%, KT361661.

The resulting topologies generated using ML and BI are generally concordant and thus only a single topology with support values generated from both analyses is presented (Fig. 8). As described in previous studies

(Yan *et al.*, 2013, 2015), the family Trachelocercidae is a monophyletic group (88% ML, 1.00 BI), being a sister clade to the family Kentrophoridae (87% ML, 1.00 BI). Within Trachelocercidae, the genus *Apotrachelocerca* occupies a basal position. The topology then separates into two clades: *Kovalevaia* and *Trachelolophos* form one clade with low support (22% ML, 0.52 BI), while *Prototrachelocerca*, *Trachelocerca* and *Tracheloraphis* form the other clade with high support (98% ML, 1.00 BI).

Tracheloraphis and *Trachelocerca* are not monophyletic as *Tracheloraphis similis* branches sister to *Prototrachelocerca* with full support and three populations of *Tracheloraphis huangi* fall within the *Trachelocerca* clade. The SSU rRNA gene sequence of *Tracheloraphis* sp. (L31520) was reported by Hirt *et al.* (1995), but without information on the morphology. It was only when Foissner & Dragesco (1996a) described the shape and structure of the oral ciliature that the generic classification based on morphology became clear. We therefore suggest that this sequence should be treated as a generic classification of an unknown environmental sequence within Trachelocercidae.

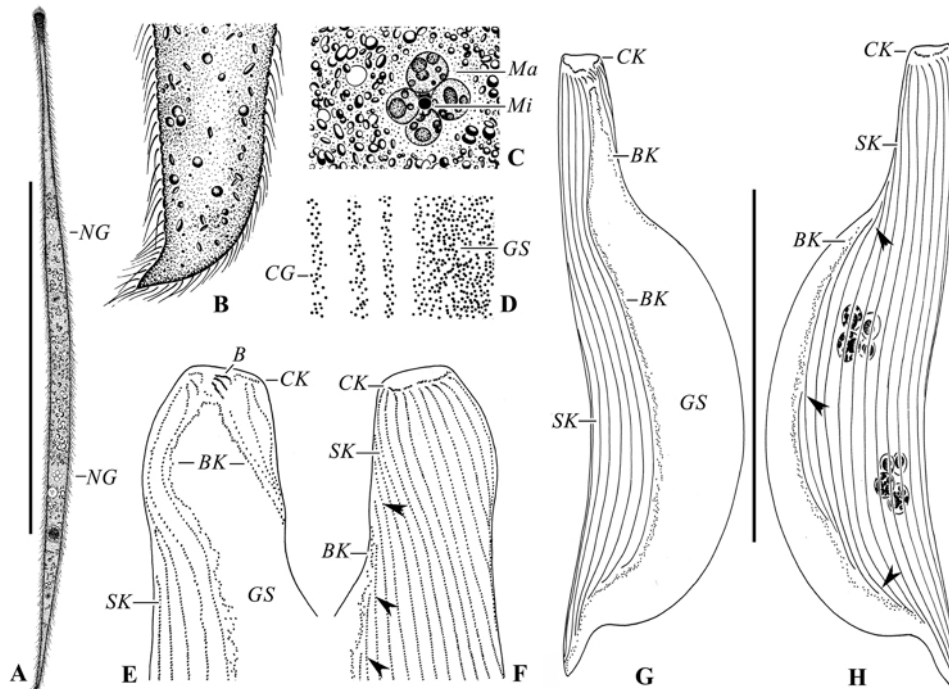


Figure 6. *Tracheloraphis similis* from life (A–D) and after protargol staining (E–H). A, typical individual; B, wedge-shaped tail; C, four macronuclei and two micronuclei forming a nuclear group; D, distribution of cortical granules between somatic kineties and in glabrous stripe; E, F, infraciliature of anterior end, indicating circumoral kinety, brosse, bristle kinety, glabrous stripe and anterior secant system forming on left side of it (arrowheads); G, H, general infraciliature, noting two nuclear groups, each of which contains about four macronuclei; arrowheads point to secant system on left side of glabrous stripe. Abbreviations: B, brosse; BK, bristle kinety; CG, cortical granules; CK, circumoral kinety; GS, glabrous stripe; Ma, macronuclei; Mi, micronuclei; NG, nuclear groups; SK, somatic kineties. Scale bars: 400 µm in A, 150 µm in G, H.

With or without *Tracheloraphis* sp. (L31520), however, the hypothesis that *Tracheloraphis* is monophyletic is rejected by the AU test ($P = 3e-033, < 0.05$ or $P = 5e-004, < 0.05$), while the hypothesis that *Trachelocerca* is monophyletic is not rejected ($P = 0.209, > 0.05$). A fuller picture of the relationship between *Trachelocerca* and *Tracheloraphis* will only be possible once more phylogenetic analyses based on multiple genetic and morphogenetic studies become available. Thus, further research needs to be performed focusing on these aspects.

EVOLUTIONARY HYPOTHESES OF TRACHELOCERCID CILIATES BASED ON MOLECULAR AND MORPHOLOGICAL EVIDENCE

As things stand, of the seven genera within the family Trachelocercidae, the SSU rRNA gene sequences of 15 species belonging to six genera are available for analysis, with the only exception being the genus *Sultanophrys*. This is sufficient to allow a new evolutionary hypothesis for the trachelocercids.

In previous studies (Foissner & Dragesco, 1996a; Foissner, 1998), it was hypothesized that the compound interrupted circumoral ciliature of *Prototrachelocerca* represents an ancient feature, as it is similar to the paroral ciliature in the loxodids, which are believed to have a common ancestor with the trachelocercids.

According to Foissner (1998), however, this hypothesis is contradicted by the fact that *Trachelocerca* has a single row of circumoral kineties, and thus possesses a less complex oral ciliature than *Prototrachelocerca*, which has a closed, uninterrupted, circumoral kinety. Foissner (1998) therefore supposed that *Trachelocerca* is the most highly evolved member within the Trachelocercidae, with the brosse feature reduced.

When the genus *Apotrachelocerca* was established (Xu *et al.*, 2011a) the situation became slightly clearer, as this genus occupies a basal position within the family according to phylogenetic analyses based on SSU rRNA gene sequences, and also possesses compound closed circumoral kineties. The molecular evidence

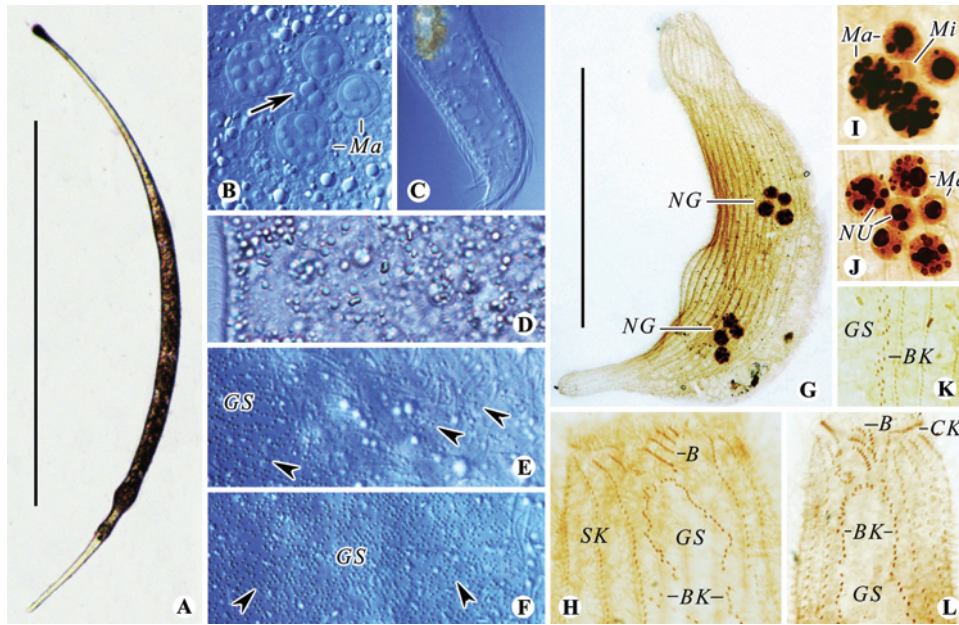


Figure 7. *Tracheloraphis similis* from life (A–F) and after protargol staining (G–L). A, typical individual; B, four macronuclei and two micronuclei forming a nuclear group; C, wedge-shaped tail; D, detailed view of middle region to show cytoplasmic granules; E, F, distribution of cortical granules (arrowheads) between somatic kineties and in glabrous stripe; G, general infraciliature, to show two nuclear groups, each of which contains about four macronuclei; H, L, infraciliature of anterior end, indicating circumoral kinety, brosse, glabrous stripe and bristle kinety; I, J, to show nuclear group including four (I) or six macronuclei (J) and micronucleus (I); K, infraciliature of mid-body portion, noting glabrous stripe bordered by bristle kinety. Abbreviations: B, brosse; BK, bristle kinety; CK, circumoral kinety; GS, glabrous stripe; Ma, macronuclei; Mi, micronucleus; NG, nuclear groups; NU, nucleolus; SK, somatic kineties. Scale bars: 400 μm in A; 150 μm in G.

therefore supports the hypothesis that the closed compound circumoral ciliature represents an ancient feature (Fig. 9). These closed compound circumoral kineties are assumed to be reduced to a single closed circumoral kinety to generate the pattern of *Trachelocerca*, which is considered to be plesiomorphic relative to the pattern of the single closed circumoral kinety with ciliary tuft that evolved in *Trachelolophos*, or the single brosse that evolved in *Kovalevaia* (Fig. 9B).

Based on morphology, the interrupted circumoral kinety patterns constitute the other evolutionary branch, with the *Prototrachelocerca* pattern group subsisting basally (Fig. 9B). The *Apotrachelocerca* pattern gives rise to the *Prototrachelocerca* pattern, with a closed compound circumoral kinety evolved to be interrupted by brosses. The *Tracheloraphis* pattern is then generated from the *Prototrachelocerca* pattern in that the compound circumoral kineties reduce to just a single one (Fig. 9B). As *Sultanophrys* has the same oral ciliature as *Tracheloraphis*, we suppose that the *Tracheloraphis* somatic infraciliature pattern produced the *Sultanophrys* pattern by orientating the

anterior secant system to the right side of the glabrous stripe (Fig. 9B).

This assumption, however, does not agree with present and previous phylogenetic studies based on SSU rRNA gene sequences (Xu *et al.*, 2011a, 2014; Yan *et al.*, 2013, 2015), in which *Trachelocerca* are always shown to branch late and cluster with *Tracheloraphis*, while *Prototrachelocerca* branches basally to them. Thus, the hypothesis based on molecular evidence is that the *Prototrachelocerca* pattern developed from the *Apotrachelocerca* pattern by the compound circumoral kineties becoming interrupted by the brosse, and then reducing to a single circumoral kinety in the *Tracheloraphis* pattern. This *Tracheloraphis* pattern then gave rise to the *Trachelocerca* pattern by the brosse becoming secondarily reduced (Fig. 9A). This molecular evidence therefore supports the hypothesis that *Trachelocerca* is the most highly evolved member within Trachelocercidae (Foissner, 1998). By contrast, *Trachelolophos* and *Kovalevaia*, which were generated from the *Apotrachelocerca* pattern with the upper circumoral kinety evolving into a ciliary tuft

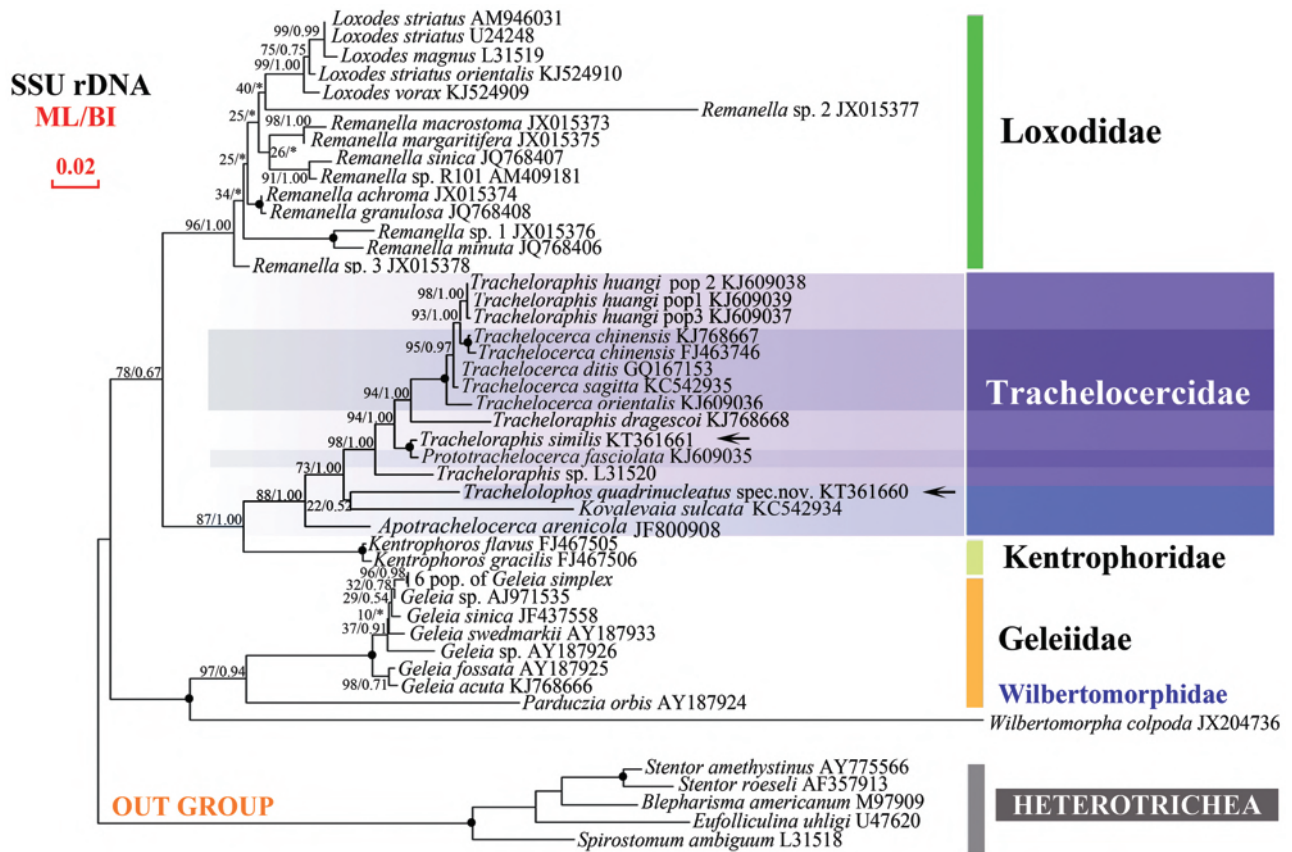


Figure 8. ML tree inferred from the SSU rDNA gene sequences showing the positions of two newly sequenced species (arrow). Nodal support for branches in the ML and BI trees are marked in order. Nodes marked by filled circles indicate full support in both analyses (100% ML, 1.00 BI). Clades with a different topology between the two analyses are shown by an asterisk. Heterotrichea are selected as outgroups. All branches are drawn to scale. The scale bar corresponds to 2 substitutions per 100 nucleotide positions.

(*Trachelolophos*) or a single brosse (*Kovalevaia*), constitute the other group in the evolutionary hypothesis (Fig. 9A). Based on the molecular evidence, we agree with the hypothesis proposed by Foissner (1997) that *Kovalevaia*, which has a keyhole-shaped circumoral kinety, represents a special evolutionary branch, sharing a common ancestor with *Trachelolophos*.

In general, therefore, we propose two evolutionary hypotheses within the family Trachelocercidae, based on either phylogenetic data or morphology evidence (Fig. 9). Both hypotheses suppose that the compound circumoral kineties shared by *Apotrachelocerca* and *Prototrachelocerca* are a plesiomorphic feature and that the single circumoral kinety in *Tracheloraphis*, *Trachelocerca*, *Trachelolophos*, *Kovalevaia* and *Sultanophrys* is synapomorphic. The main contradiction between these two hypotheses concerns the position of *Trachelocerca*, i.e. whether the simple circumoral

kinety with no brosse feature is ancestral or secondarily reduced. Unfortunately, more evidence including both gene sequences and morphogenetic data is needed to reach a more certain conclusion.

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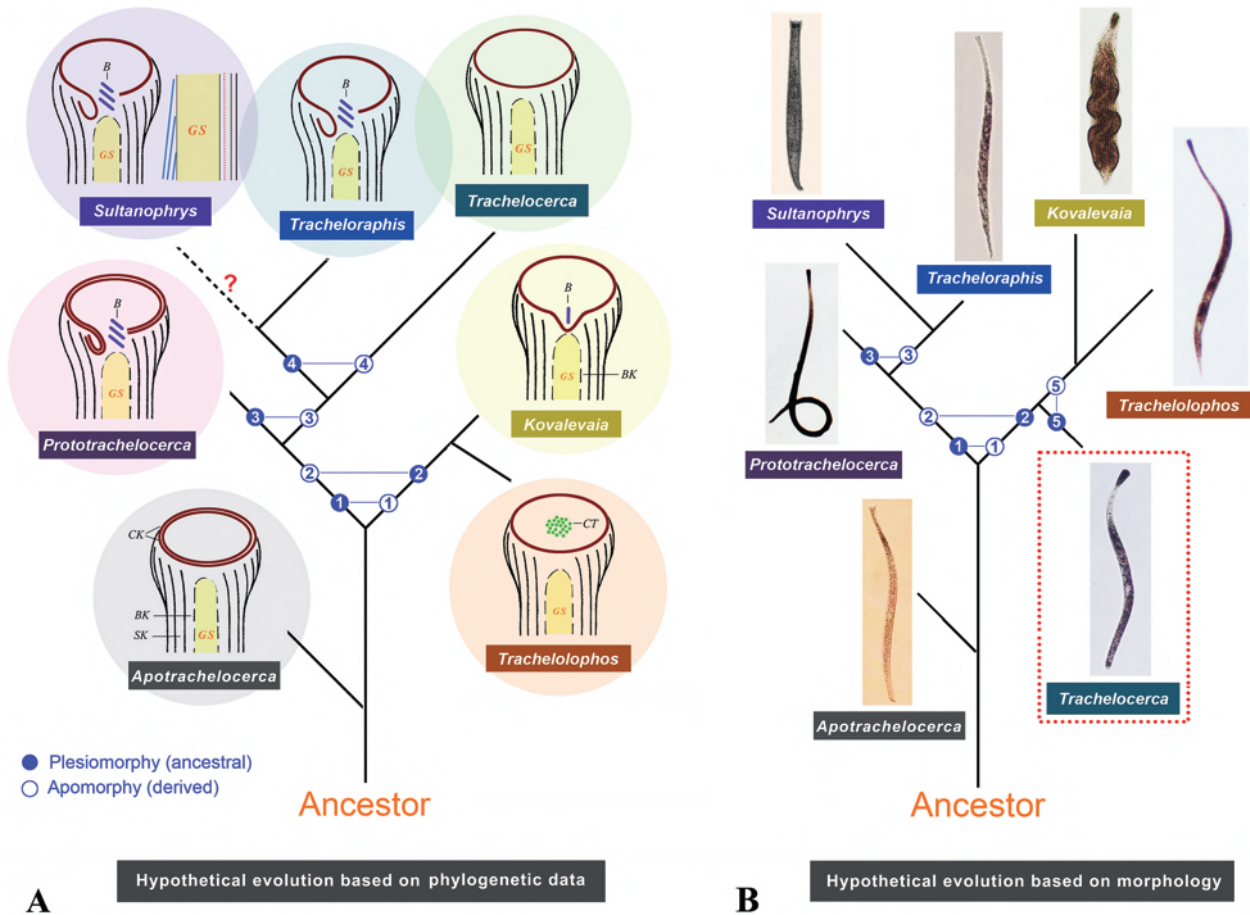


Figure 9. Hypothetical evolution of trachelocercids based on the scenario shown in Figure 8 (A) or according to oral ciliature pattern (B). The question mark refers to a possible evolutionary path as no molecular data are available for the genus *Sultanophrys*. The main difference between cladogram A and B is the position of *Trachelocerca*, which is marked with a frame. The characters used in estimation of the evolutionary relationship are listed in Table 2. Abbreviations: B, brosse; BK, bristle kinety; CK, circumoral kinety; CT, ciliary tuft; GS, glabrous stripe; SK, somatic kineties.

Table 2. Morphological characters used in Figure 9

● Plesiomorph	○ Apomorph
1 Circumoral kineties composed of two rows of dikinetids	Circumoral kinety composed of one row of dikinetids
2 Circumoral kinety uninterrupted	Circumoral kineties interrupted at brosse cleft
3 Circumoral kineties composed of two rows of dikinetids	Circumoral kinety composed of one row of dikinetids
4 Circumoral kineties interrupted at brosse cleft	Circumoral kinety uninterrupted
5 Oral ciliature composed of circumoral kinety only	Oral ciliature composed of circumoral kinety and brosse or ciliary tuft

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