Redescriptions of three trachelocercid ciliates (Protista, Ciliophora, Karyorelictea), with notes on their phylogeny based on small subunit rRNA gene sequences

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Three trachelocercid ciliates, Kovalevaia sulcata (Kovaleva, 1966) Foissner, 1997, Trachelocerca sagitta (Müller, 1786) Ehrenberg, 1840 and Trachelocerca ditis (Wright, 1982) Foissner, 1996, isolated from two coastal habitats at Qingdao, China, were investigated using live observation and silver impregnation methods. Data on their infraciliature and morphology are supplied. The small subunit rRNA (SSU rRNA) genes of K. sulcata and Trachelocerca sagitta were sequenced for the first time. Phylogenetic analyses based on SSU rRNA gene sequence data indicate that both organisms, and the previously sequenced Trachelocerca ditis, are located within the trachelocercid assemblage and that K. sulcata is sister to an unidentified taxon forming a clade that is basal to the core trachelocercids.

INTRODUCTION

Karyorelicteans are an enigmatic group of ciliates that form a highly specific component of the marine sand microbiota (Foissner, 1997a). Prior to the development of reliable methods for their fixation and histological staining, morphological analyses of karyorelicteans were limited by their extreme fragility. Nevertheless, features such as body shape, nuclear structure and somatic cortical ultrastructure were reported in several pioneering studies (Dragesco, 1960; Dragesco & Dragesco-Kerneis, 1986; Raikov et al., 1975; Wilbert, 1986). Trachelocercidae Kent, 1881 is the largest family within the class Karyorelictea Corliss, 1974 and is commonly represented in marine littoral sands (Al-Rasheid, 1996, 1997, 1998, 2001; Al-Rasheid & Foissner, 1999; Foissner & Dragesco, 1996a). Based mainly on observations of specimens in vivo, about 70 species of trachelocercids have traditionally been recognized (Dragesco, 1960; Raikov et al., 1975; Wilbert, 1986; Carey, 1992). However, detailed knowledge of their infraciliature was lacking (Xu et al., 2011b). Following the development of reliable methods for cell fixation, Foissner & Dragesco (1996a) established a new standard for generic classification of trachelocercids based mainly on the infraciliature and in particular the oral infraciliature.

Furthermore, it was demonstrated that several characters previously used for genus-level circumscription, such as the width of the glabrous stripe and the shape of the tail, are of limited value because of the presence of transitional character states (Foissner & Dragesco, 1996b).

In recent years several detailed studies of karyorelicteans have been carried out using modern methods such as protargol impregnation and scanning electron microscopy (Foissner, 1996, 1997b; Foissner & Dragesco, 1996a, b; Dragesco, 1997, 1999; Xu et al., 2012). However, molecular data on trachelocercids remain limited. To date, only 35 small subunit (SSU) rRNA gene sequences are available for karyorelicteans, including 16 of trachelocercids (Xu et al., 2013a). Previous studies have shown that the class Karyorelictea is a well-defined monophyletic group that is divided into four clades corresponding to the four families: Loxodidae, Geleiidae, Kentrophoridae and Trachelocercidae (Xu et al., 2011c). According to Xu et al. (2011c), the family Trachelocercidae is also monophyletic. Among the seven genera of Trachelocercidae, SSU rRNA gene sequence data are available for only three: Trachelocerca, Tracheloraphis and Apotrachelocerca.

In the present study, morphological data on three trachelocercids isolated from marine coastal habitats at Qingdao, China, are documented based on observations of specimens in vivo and following protargol impregnation. In addition, the SSU rRNA genes of Kovalevaia sulcata and
Trachelocerca sagitta were sequenced for the first time and phylogenetic analyses were performed to assess the systematic positions of these two species within the family Trachelocercidae.

METHODS

K. sulcata was collected on 2 September 2009 from a small puddle near mariculture ponds in Qingdao, northern China (36° 21’ N 120° 40’ E), where the water temperature was 28 °C and salinity was 20%. Both Trachelocerca sagitta and Trachelocerca ditis were isolated from the intertidal zone of the Diaosuyuan sandy beach at Qingdao, China (36° 5’ N 120° 27’ E). Trachelocerca sagitta was collected on 3 September 2009, when the water temperature was 28 °C and salinity was 13.5%. Trachelocerca ditis was collected on 7 May 2011, when the water temperature was about 20 °C and salinity was 17%. Sampling methods were mainly according to Xu et al. (2012). The SSU rRNA gene cloning and sequencing were performed according to methods described by Gao (2012). The universal eukaryotic primers Euk A (5’-TGATCCTTCTGCAGGTTCACCTAC-3’) and Euk B (5’-TGATCCTTCTGCAGGTTCACCTAC-3’) were used for amplification (Elwood et al., 1985).

Eight cells each of K. sulcata and Trachelocerca sagitta were isolated for SSU rRNA gene sequencing. DNA extraction, PCR amplification, SSU rRNA gene cloning and sequencing were performed according to methods described by Gao et al. (2012). The universal eukaryotic primers Euk A (5’-AACCTGTTGTACCTGCGCAT-3’) and Euk B (5’-TGATCCTTCTGCAGGTTCACCTAC-3’) were used for amplification (Elwood et al., 1985).

RESULTS AND DISCUSSION

Kovalevaia sulcata (Kovaleva, 1966) Foissner, 1997 (Figs 1 and 2; Table 1)

Description of Qingdao population. Extended cell about 800–1250 µm × 25 µm in vivo; body flattened ribbon-like, flexible and contractile (Figs 1a, b and 2a, b, d); head conspicuous, triangular in shape (Figs 1a and 2a); posterior region narrowing sharply to form a short tail-like process (Figs 1a, b and 2a, b). Cells dark brownish at low magnification due to large brown cortical granules (Fig. 2a, b, d); glabrous stripe on left side of body, with narrow longitudinal groove, extends entire body length and appears as a bright line at low magnification (Figs 1b, f and 2b, f). Two types of cortical granules: type 1 large, ca. 1 µm in diameter, brown, scattered between ciliary rows but absent from longitudinal groove (Figs 1f, g and 2c, f, g); type 2 small, ca. 0.5 µm in diameter, colourless, scattered both between ciliary rows and within glabrous stripe (Figs 1f, g and 2c, f, g). The SSU rRNA gene sequences were aligned using CLUSTAL W v.1.80 (Thompson et al., 1994) and refined using BioEdit v.7.0.5 (Hall, 1999) to excise (i.e. mask) highly variable regions. Bayesian inference (BI) was performed with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) using the GTR+I+G model as selected by Akaike information criterion in MrModeltest v.2.0 (Nylander, 2004). The chain length for our analysis was 1 000 000 generations with trees sampled every 100 generations. The first 25% of sampled trees were discarded as burn-in (Zhang et al., 2012). Maximum-likelihood (ML) analyses were carried out online on the CIPRES Science Gateway (CIPRES Portals: http://www.phylo.org/sub_sections/portal) with RAxML-HPC BlackBox (7.2.7) (Stamatakis et al., 2008). The reliability of internal branches was estimated by bootstrapping with 1000 replicates.

Fig. 1. Kovalevaia sulcata from life (a–g) and after protargol impregnation (h–k). (a) Typical individual. (b) Contracted individual. (c, d) Shape variants. (e) Mid-region of cell marking macronuclei and ellipsoid (crystalline?) inclusions (arrowhead). (f, g) Distribution of large and small cortical granules in glabrous stripe (f), and between ciliary rows (g), showing longitudinal groove (arrowhead). (h, i) Left (h) and right (i) side view of anterior body region, indicating glabrous stripe, somatic kineties, bristle kinety, brossie and continuous (uninterrupted) circumoral kinety consisting of one row of dikinetids. (j, k) Infraciliature of left (j) and right (k) side, showing that the left side is almost completely occupied by the glabrous stripe. B, brossie; BK, bristle kinety; CK, circumoral kinety; GS, glabrous stripe; LCG, large cortical granules; LG, longitudinal groove in centre of glabrous stripe; Ma, macronuclei; SCG, small cortical granules; SK, somatic kineties. Bars: 400 µm (a, j, k), 200 µm (b–d), 30 µm (h, i).
Cytoplasm colourless, packed with colourless ellipsoid granules, 1–3 μm long (Fig. 1e). Locomotion by gliding over sand grains and organic debris. Cell surface densely ciliated apart from glabrous stripe on left side of body (Figs 1j and 2j). Entire infraciliature consisting of dikinetids (Figs 1j, k and 2h–k). Nine to 13 somatic kineties in head region, 10–13 in trunk (Fig. 1j, k). Somatic cilia about 7–8 μm long. Glabrous stripe bordered by the bristle kinety (Figs 1h and 2j). Anterior and posterior secant system formed on left side of glabrous stripe where some kineties abut bristle kinety (Fig. 2j). Oral infraciliature consisting of a keyhole-shaped circumoral kinety and a minute brossse (Figs 1h, i and 2h, i). Seventeen to 39 globular macronuclei, ca. 5–12 μm in diameter, containing many small nucleoli and forming a long strand in cell median (Figs 1c–e, j and 2e, k). Five to 12

Table 1. Morphometric data (μm) for Kovalevaia sulcata (first line), Trachelocerca sagitta (second line) and Trachelocerca ditis (third line)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length</td>
<td>281</td>
<td>1204</td>
<td>487.0</td>
<td>43.3</td>
<td>25</td>
</tr>
<tr>
<td>Body width</td>
<td>70</td>
<td>200</td>
<td>115.4</td>
<td>24.4</td>
<td>26</td>
</tr>
<tr>
<td>SK on head (no.)</td>
<td>9</td>
<td>13</td>
<td>10.6</td>
<td>9.4</td>
<td>13</td>
</tr>
<tr>
<td>SK on trunk (no.)</td>
<td>10</td>
<td>13</td>
<td>11.9</td>
<td>6.2</td>
<td>23</td>
</tr>
<tr>
<td>Distance from anterior end of body to Ma</td>
<td>88</td>
<td>303</td>
<td>148.1</td>
<td>34.1</td>
<td>20</td>
</tr>
<tr>
<td>Ma (no.)</td>
<td>17</td>
<td>39</td>
<td>27.8</td>
<td>21.3</td>
<td>28</td>
</tr>
</tbody>
</table>

All data are based on protargol-impregnated specimens. CV, Coefficient of variation (%); Ma, macronuclei; n, number of specimens investigated; sk, somatic kineties.
Table 2. Comparison of Kovalevaia sulcata and Trachelonema sulcata populations

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>K. sulcata</th>
<th>K. sulcata</th>
<th>T. sulcata</th>
<th>T. sulcata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Qingdao population</td>
<td>Roscoff population</td>
<td>Black Sea population</td>
<td>Posjet Gulf population</td>
</tr>
<tr>
<td>Length in vivo (µm)</td>
<td>800–1250</td>
<td>800–1200</td>
<td>600–1200</td>
<td>–</td>
</tr>
<tr>
<td>Width in vivo (µm)</td>
<td>25</td>
<td>50</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Somatic kineties</td>
<td>10–13</td>
<td>11–18</td>
<td>18–24</td>
<td>10–16</td>
</tr>
</tbody>
</table>

–, Data not available.

micronuclei, ca. 3–5 µm in diameter, distributed between macronuclei.

Remarks. K. sulcata was originally isolated from the Black Sea by Kovaleva (1966) who assigned it to the genus Trachelonema. It was then redescribed by Raikov & Kovaleva (1968) based on a population from Posjet Gulf (Japan Sea). Foissner (1997a) made a detailed study based on the population collected at Roscoff (France) following both protargol impregnation and scanning electron microscopy, and established the new genus Kovalevaia for K. sulcata because its circumoral kinety surrounds not only the head but also the brosse cleft. The Qingdao population corresponds well with both the Roscoff and the Black Sea populations in terms of body size and shape, the width of the glabrous stripe and the number of macronuclei (Kovaleva, 1966; Foissner, 1997a). The only noteworthy difference is the number of somatic kineties (10–13 in the Qingdao population versus 11–18 in the Roscoff population and 18–24 in the Black Sea population) (Table 2). According to Foissner (1997a), such differences are population-dependent as each of these values overlaps with at least one other. Consequently, we identified the Qingdao isolate as a population of K. sulcata.

Trachelocerca sagitta (Müller, 1786) Ehrenberg, 1840 (Figs 3 and 4; Table 1)

Description of Qingdao population. Extended cell about 220–500 µm × 15–35 µm in vivo; body ribbon-like, flexible and contractile (Figs 3a, b, d and 4a, b); head conspicuous, triangular in shape; neck clearly distinguishable from trunk (Figs 3a and 4a). Cells greyish at low magnification in bright-field microscopy (Fig. 4a, b). Cortical granules ca. 0.5–1 µm in diameter, colourless, mainly distributed within glabrous stripe and scattered between ciliary rows (Figs 3c and 4d, e). Cytoplasm colourless, packed with ellipsoid, colourless granules. Locomotion by gliding over sand grains and organic debris.

Infraciliature consisting entirely of dikinetids (Fig. 3h, i). Nine to 12 somatic kineties in head region (Figs 3f, g and 4f, g), 11–14 in trunk region (Fig. 3h, i). Anterior and posterior secant system formed on left side of glabrous stripe where some kineties abut bristle kinety (Fig. 3g). Glabrous stripe bordered by bristle kinety (Figs 3g, i and 4f). Oral infraciliature comprising a single circumoral kinety (Figs 3f–i and 4f, g). Usually 3–6 globular macronuclei and two micronuclei forming a nuclear group located in mid-region of cell (Figs 3a, b, e, i and 4a).

Fig. 3. Trachelocerca sagitta from life (a–e) and after protargol impregnation (f–i). (a) Typical individual. (b) Contracted individual. (c) Distribution of cortical granules between ciliary rows (arrows) and in glabrous stripe (arrowheads). (d) Shape variants. (e) Macronuclei. (f, g) Right (f) and left (g) side view of anterior body region, indicating bristle kinety, somatic kineties, continuous (uninterrupted) circumoral kinety consisting of one row of dikinetids, and anterior secant system (arrowheads). (h, i) Infraciliature of right (h) and left (i) side. BK, bristle kinety; CK, circumoral kinety; GS, glabrous stripe; Ma, macronuclei; SK, somatic kineties. Bars: 400 µm (a, d, h, i), 200 µm (b), 50 µm (f, g).
Macronuclei usually containing several small nucleoli.

**Remarks.** *Trachelocerca sagitta* was originally reported by Müll (1786) as *Vibrio sagitta*. Ehrenberg (1840) established a new genus, *Trachelocerca*, for this species. However, no type species was fixed so the genus remained invalid until Foissner & Dragesco (1996a) deposited in the Oberösterreichisches Landesmuseum, Linz, Austria, two neotype slides with specimens of *Trachelocerca sagitta* from Roscoff. The Qingdao population corresponds well with the Roscoff population (Foissner & Dragesco, 1996a) in terms of body shape and the number of somatic kineties (Table 3), the only difference being the body length (220–500 μm versus about 1000 μm in the Roscoff population). The Qingdao population also agrees well both with the original population (Müll, 1786) in terms of the number of somatic kineties, and with a population from South Wales (Wright, 1982) in terms of the range of body size and the number of somatic kineties. As the body length of this species is variable among these reports, we believe this feature is population-dependent and that the Qingdao isolate is a population of *Trachelocerca sagitta*.

**Trachelocerca ditis** (Wright, 1982) Foissner, 1996 (Figs 5 and 6; Table 1)

**Description of Qingdao population.** Cells *in vivo* about 450–700 μm × 20–30 μm, slender, highly flexible and contractile (Figs 5a–c and 6a–c); neck elongate and easily distinguishable from trunk (Figs 5a and 6a, b). Cells greyish at low magnification in bright-field microscopy (Fig. 6a–c). Cortical granules about 0.8 μm across, colourless, distributed both between ciliary rows and within glabrous stripe where they are densely packed (Figs 5d and 6g, i). Cytoplasm colourless, packed with food vacuoles and colourless ellipsoidal granules 1–3 μm in size. Locomotion by gliding over sand grains and organic debris.

Entire infraciliature consisting of dikinetids (Fig. 5g, h). Cilia about 10–11 μm long *in vivo*. Glabrous stripe narrow, usually about the width of three ciliary rows (Figs 5g and

| Table 3. Comparison of *Trachelocerca sagitta* and *Tracheloraphis conformis* populations |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Characteristic                  | *T. sagitta*    | *T. sagitta*    | *Vibrio sagitta* | *Tracheloraphis conformis* |
| Length *in vivo* (μm)           | Qingdao population | Roscoff population | Original population | South Wales population |
| Width *in vivo* (μm)            | 220–500         | 1000            | —               | 300–800          |
| Somatic kineties               | 15–35           | 30              | —               | —               |
|                                 |                 |                 |                 |                  |

—, Data not available.
Eleven to 15 somatic kineties in head region (Figs 5e, f and 6d, h), 16–22 in trunk (Figs 5g, h and 6h). Anterior and posterior secant systems formed on left side of glabrous stripe where some kineties abut bristle kinety (Figs 5e, g and 6h). Glabrous stripe bordered by bristle kinety (Figs 5e, g and 6h). Oral infraciliature comprising a single circumoral kinety (Figs 5e–h and 6h). Four to 12 (usually four) globular macronuclei and two micronuclei forming a nuclear group located in mid-region of cell (Figs 5a, c and 6f).

Remarks. *Trachelocerca ditis* was first described by Wright (1982) who assigned it to the genus *Tracheloraphis*. Following a detailed examination of a population of *Tracheloraphis ditis* from Roscoff, Foissner & Dragesco (1996a) transferred it to the genus *Trachelocerca* as it lacks a brosse. The Qingdao population of *Trachelocerca ditis* corresponds well with the Roscoff population (Foissner & Dragesco, 1996a) in terms of its body shape and the width of the glabrous stripe (Table 4). The main differences are the range of body length (450–700 µm versus 800–1000 µm in the Roscoff population) and the number of somatic kineties (16–22 versus 24–33 in the Roscoff population). Compared with the populations of South Wales (Wright, 1982) and one previously described from Qingdao (Mazei et al., 2009).
we believe these features to be population-dependent and, given their strong similarity in other respects (Table 4), we conclude that this Qingdao isolate is a population of *Trachelocerca ditis*.

### Table 4. Comparison of *Trachelocerca ditis* populations

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>T. ditis</em> Qingdao population (1)</th>
<th><em>T. ditis</em> Roscoff population</th>
<th><em>T. ditis</em> South Wales population</th>
<th><em>T. ditis</em> Qingdao population (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length <em>in vivo</em> (µm)</td>
<td>450–700</td>
<td>800–1000</td>
<td>300–800</td>
<td>−</td>
</tr>
<tr>
<td>Width <em>in vivo</em> (µm)</td>
<td>20–30</td>
<td>40–50</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Somatic kineties</td>
<td>16–22</td>
<td>24–33</td>
<td>18–22</td>
<td>21–26</td>
</tr>
</tbody>
</table>

−, Data not available.

### Phylogenetic positions of *K. sulcata* and *Trachelocerca sagitta* (Fig. 7)

The BI and ML trees have similar topologies and therefore only the ML tree is presented here (Fig. 7). The four
karyorelictean families, Geleidiidae Loxodidae, Trachelocercidae and Kentrophoridae, form four monophyletic groups with high support values (96% ML, 0.93 BI; 99% ML, 1.00 BI; 82% ML, 0.93 BI; and 98% ML, 1.00 BI, respectively). Within the family Trachelocercidae, Apotrichelocerca arenicola (brosse absent; uninterrupted compound circumoral cirri) occupies the basal position, with moderate statistical support (82% ML, 0.93 BI). The next most deeply branching taxa are K. sagitta (with brosse; uninterrupted, simple, keyhole-shaped circumoral kinety) and an unidentified trachelocercid (GenBank accession no. AJ971525) that together form a moderately well-supported clade (82% ML, 0.91 BI). Compared with Tracheloraphis (with brosse that interrupts the simple circumoral kinety), Trachelocerca sagitta and Trachelocerca ditis (brosse absent; uninterrupted simple circumoral kinety) fall into a higher position in the phylogenetic trees (88% ML, 1.00 BI). These findings suggest that the compound circumoral ciruature in Aptomatchelocerca may be the ancestral character state in trachelocercids, whereas the simple, uninterrupted circumoral ciruature in Trachelocerca is a derived character state, which is consistent with the hypothesis proposed by Foissner (1997a). However, to test this hypothesis more rigorously, and to better resolve phylogenetic relationships within the Trachelocercidae, gene sequences for more taxa, particularly of the genera Prototrichelocerca, Trachelolophos and Sultanophris, are required.

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