Parabirojimia multinucleata spec. nov. and *Anteholosticha scutellum* (Cohn, 1866) Berger, 2003, marine ciliates (Ciliophora, Hypotrichida) from tropical waters in southern China, with notes on their small-subunit rRNA gene sequences

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Few studies using modern methods have been carried out on ciliated protozoa in tropical marine waters. In the present work, two hypotrichs, *Parabirojimia multinucleata* spec. nov. and *Anteholosticha scutellum* (Cohn, 1866) Berger, 2003, collected from Daya Bay in southern China, were investigated morphologically. *P. multinucleata* is distinguished by the following combination of characters: slender body, without a snout-like protrusion in the frontal field, and about 50 macronuclear nodules. The poorly known *A. scutellum* has never been investigated using modern methods; hence, a redescription is needed. During the present study, observations of specimens *in vivo* and following protargol impregnation revealed new information concerning structures such as the cortical granules and the infraciliature. A redescription and improved diagnosis are supplied based on the China population. The small-subunit (SSU) rRNA gene was sequenced for both organisms and comparisons with those of similar congeners clearly support the findings based on morphological studies.

INTRODUCTION

The hypotrichs are a species-rich and morphologically diverse group of ciliates. Many marine hypotrichous species have not been adequately investigated, and their identification remains problematic. Much of this confusion exists because earlier studies provided descriptions only of morphology, without accurate information on the infraciliature and without adequate morphometric data, leaving the validity of many species uncertain. A typical example of such a species is *Anteholosticha scutellum* (Cohn, 1866) Berger, 2003. In recent years, this situation has improved, with the routine application of silver impregnation methods when describing new taxa (Borror & Wicklow, 1983; Berger, 2004, 2006; Hu *et al.*, 2002; Li *et al.*, 2008; Lin

et al., 2008; Lynn & Small, 2002; Shao *et al.*, 2008a, b; Hu, 2008).

The genus *Parabirojimia* was established by Hu *et al.* (2002) and is characterized as follows: adoral zone of membranelles (AZM) bipartite; frontal cirri clearly differentiated; buccal cirrus (cirri) and transverse cirri present; frontoterminal and caudal cirri absent; midventral complex composed of midventral pairs and one or more midventral rows at posterior end of complex; one left and two or more right marginal rows which derive from individual anlagen within each parental row; transverse cirri originate within frontal-midventral-transverse cirri anlagen and from part of the right marginal cirri anlagen. *Parabirojimia similis* Hu *et al.*, 2002 is the only described species of this genus.

In the spring of 2008, during a survey of the ciliate fauna in coastal waters of Daya Bay in southern China, we isolated two hypotrichs. One appeared to represent a novel species of the genus *Parabirojimia*, whereas the other was *Anteholosticha scutellum*, a poorly known form which was originally described by Cohn (1866) as *Oxytricha scutellum*.

Abbreviations: AZM, adoral zone of membranelles; BI, Bayesian inference; ML, maximum likelihood; MP, maximum parsimony; SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the SSU rRNA gene sequences of *Parabirojimia multinucleata* and *Anteholosticha scutellum* are FJ156104 and FJ156105.

The morphology and infraciliature of these two species together with their small-subunit (SSU) rRNA gene sequences are described here.

METHODS

The two hypotrichs were collected on 18 March 2008 from Daya Bay (22° 36' 03" N 114° 32' 46" E), on the South China Sea coast of southern China. The water temperature was about 25 °C, salinity about 28‰ and pH about 7.8. *Parabirojimia multinucleata* was collected from the surface of a sea squirt species (*Pyura* sp.) in mussel-culturing waters. *Anteolosticha scutellum* was collected from a sandy beach by sampling the upper 15 cm layer of sand together with water from the site and maintaining as raw cultures for 1 week at room temperature.

Live cells were observed with differential interference contrast microscopy and their infraciliature was revealed using the protargol impregnation method according to Wilbert (1975). Living individuals were examined and measured at ×1000 magnification; counts, measurements and drawings of stained specimens were performed at ×1250 magnification with the aid of a camera lucida (Lin *et al.*, 2007). Terminology is mainly according to Corliss (1979) and Berger (2006).

Genomic DNA extraction, PCR amplification and SSU rRNA gene cloning and sequencing were performed according to Gong *et al.* (2007) and Yi *et al.* (2008). The sequence of *P. similis* (GenBank accession no. DQ503584) was used for comparison with *P. multinucleata*. The nucleotide sequences of four other *Anteolosticha* species were used for comparative purposes: *Anteolosticha manca* (GenBank accession no. DQ503578), *Anteolosticha multistilata* (AJ277876), *Anteolosticha* sp1-QD-07060701 (FJ775713) and *Anteolosticha* sp2-QD-04082401 (EF123707). Other sequences used in this study were obtained from the NCBI/GenBank database. Phylogenetic trees were constructed according to the methods reported by Gao *et al.* (2008).

Briefly, the program MRMODELTEST version 2 (Nylander, 2004) selected GTR+I+G under the AIC as the best model, which was

then used for Bayesian and maximum-likelihood (ML) analysis. A Bayesian inference (BI) was performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and the program was run for 1 000 000 generations with a sample frequency of 100 and a burn-in of 2500. An ML tree was constructed with the PhyML program version 2.4.4 (Guindon & Gascuel, 2003) with 1000 bootstrap replicates. A maximum-parsimony (MP) tree was produced with PAUP* 4.0b10 (Swofford, 2002) by bootstrapping with 1000 replicates.

RESULTS

Parabirojimia multinucleata spec. nov. (Figs 1 and 2; Tables 1 and 2)

Diagnosis. Marine *Parabirojimia*, 150–350 × 30–60 µm *in vivo*; body outline elongate elliptical; AZM bipartite with 46–60 membranelles in total; five right and one left marginal rows; three frontal, one buccal and six to 11 transverse cirri; midventral complex composed of about five cirral pairs and one long ventral row of 44–69 cirri; three dorsal kineties; about 50 macronuclear nodules.

Etymology. This species has about 50 macronuclear nodules; therefore, its name recalls this feature: multi-(many), nucleate.

Type locality. Mussel-culturing waters in Daya Bay (22° 36' 03" N 114° 32' 46" E), Guangdong, south China; salinity approx. 28‰, water temperature approx. 25 °C, approx. pH 7.8.

Fig. 1. *Parabirojimia multinucleata* spec. nov. observed *in vivo* (a–d) and after protargol impregnation (e–g). (a) Ventral view of a typical individual. (b) Lateral view. (c) Dorsal side, showing the tiny cortical granules and the irregular-shaped larger granules (arrows). (d) Variations in cell shape illustrating the highly flexible nature of the cell. (e) Ventral infraciliature of the anterior portion of the cell. (f, g) Ventral (f) and dorsal (g) views showing the infraciliature. Arrow in (g) indicates the transverse cirri; arrowheads in (g) mark the micronuclei. AZM, Adoral zone of membranelles; BC, buccal cirri; DK, dorsal kineties; E, endoral membrane; FC, frontal cirri; FM, frontal adoral membranelles; LMC, left marginal cirri; LMR, row of left marginal cirri; Ma, macronucleus; MVC, midventral cirri; P, paroral membrane; RMC, right marginal cirri; RMR, rows of right marginal cirri; VM, ventral adoral membranelles; VR, ventral cirral row. Bar, 100 µm.

Fig. 2. Photomicrographs of *Parabirojimia multinucleata* spec. nov. from life (a–d, f, g) and after protargol impregnation (e, h–k). (a) Ventral view of a typical individual. (b) A slender individual. (c, d) Ventral views of the anterior portion of the cell; arrowheads in (d) mark the frontal cirri. (e) Infraciliature of oral field. (f, g) Dorsal views, showing the spindle-shaped granules (arrowheads in f) and the tiny cortical granules (arrows in g). (h) Ventral view to show the general infraciliature. (i) Macronuclei and micronuclei (arrowheads). (j) Dorsal kineties. (k) Posterior portion of the cell; arrow marks the transverse cirri, arrowhead depicts a micronucleus. Bars, 100 µm (a–c, h), 50 µm (d, e, j, k) and 10 µm (f, g, i).

Type location. One holotype slide of protargol-impregnated specimens is deposited in the Laboratory of Protozoology, OUC, China, with registration number 2008:6:1:1. One paratype slide is deposited in the collection of the Natural History Museum, London, UK, with registration number 2008:5:13:1.

Description. Body slender and band-like, flexible, mostly about 300×50 µm *in vivo*, ratio of length to width 5–6:1; dorsoventrally flattened approx. 3:2 (Fig. 1b). Body widest in anterior quarter, posteriorly gradually narrowed (Figs 1a, d and 2a, b). Buccal field about 10% of body length. AZM bipartite, distal end bending posteriorly onto right side, making an inconspicuous furrow along anterior-right margin.

Pellicle thin and flexible. Two types of cortical granules: type I colourless and small, approx. 0.2 µm across, sparsely arranged on dorsal side (Figs 1c and 2g); type II spindle-shaped, about 2×1.5 µm in size, greyish when observed at high magnification, sparsely distributed and few in number, hence never render cell any colour (Figs 1c and 2f). Cytoplasm opaque and colourless, often dark grey or even black in central part of body due to food vacuoles (8–20 µm across) and other contents (Figs 1a and 2a, c), often containing numerous light-reflecting globules (2–3 µm across). About 50 macronuclear nodules, oval to elongate in shape and difficult to observe *in vivo* (Figs 1g and 2h, i). Six to 12 spherical micronuclei (Mi), each about 3 µm across (Figs 1g and 2i, k).

Locomotion by slow crawling without pause on debris or on bottom of Petri dish. Conspicuously flexible.

AZM with a large gap between the two parts; bases of membranelles in anterior part clearly shorter than those in posterior part (Figs 1e, f and 2e). Cilia in anterior part

about 20 µm long, whereas those in posterior portion are much shorter. Paroral and endoral membranes about equal in length, slightly curved and noticeably crossed (Fig. 1e, f).

All cirri relatively fine, mostly about 10 µm long; each cirral base appears to be composed of two rows of basal bodies; transverse cirri about 15 µm long. Invariably three slightly enlarged frontal cirri, each about 20 µm long, which are continuous with the midventral cirral pairs; single buccal cirrus situated near intersection of undulating membranes (Fig. 1e, f); midventral complex composed of about five cirral pairs and one midventral row composed of 55 cirri and extending to about 65% of body length; six to 11 inconspicuous transverse cirri that project only slightly beyond posterior body margin (Figs 1f and 2a, k). One left (LMR) and five right (RMR) marginal rows; RMRs obliquely orientated from anterior right to posterior left; leftmost two or three RMRs posteriorly shortened (Fig. 1f, g). Three complete dorsal kineties, dorsal cilia about 5 µm long (Figs 1g and 2j).

SSU rRNA gene sequence (Fig. 3). The GenBank/EMBL/DDBJ accession number for the SSU rRNA gene sequence of *Parabirojimia multinucleata* is FJ156104 (1773 bp). It differs from that of *P. similis* in 23 sites (Fig. 3b). It grouped with *P. similis* with full support in all the methods performed (BI/ML/MP, 1.00/100/100).

Remarks and comparison

Previously, *Parabirojimia* was a monotypic genus, the only species being *Parabirojimia similis* Hu *et al.*, 2002. The novel species differs from *P. similis* in having more macronuclear nodules (43–70 vs 3–6), no snout-like protrusion in the frontal field (present in *P. similis*), invariably five RMRs (five to eight in *P. similis*) and more

Table 1. Morphometric characterization of *P. multinucleata* spec. nov. and *A. scutellum*

Data are based on protargol-stained specimens.

Character	Species	Min.	Max.	Mean	SD	n
Body length (µm)	<i>P. multinucleata</i>	190	320	246.8	30.9	25
	<i>A. scutellum</i>	55	70	60.5	4.4	12
Body width (µm)	<i>P. multinucleata</i>	55	90	73.4	8.0	25
	<i>A. scutellum</i>	28	40	35.7	4.1	12
Buccal field length (µm)	<i>P. multinucleata</i>	45	60	51.9	4.4	25
	<i>A. scutellum</i>	20	25	22.3	1.5	12
Body length/buccal field length	<i>P. multinucleata</i>	4	6	4.7	0.4	25
	<i>A. scutellum</i>	2	3	2.7	0.2	12
Adoral membranelles (n)	<i>P. multinucleata</i>	46	60	52.4	3.7	25
	<i>A. scutellum</i>	17	18	17.6	0.5	12
Buccal cirri (n)	<i>P. multinucleata</i>	1	1	1.0	0	25
	<i>A. scutellum</i>	1	1	1.0	0	12
Frontal cirri (n)	<i>P. multinucleata</i>	2	2	2.0	0	25
	<i>A. scutellum</i>	3	3	3.0	0	12
Frontoterminal cirri (n)	<i>P. multinucleata</i>	–	–	–	–	–
	<i>A. scutellum</i>	2	2	2.0	0	12
Midventral cirri (n)	<i>P. multinucleata</i>	6	16	9.9	2.0	25
	<i>A. scutellum</i>	12	14	13.0	0	12
Transverse cirri (n)	<i>P. multinucleata</i>	6	11	8.3	1.1	25
	<i>A. scutellum</i>	8	8	8.0	0	12
Pretransverse ventral cirri (n)	<i>P. multinucleata</i>	–	–	–	–	–
	<i>A. scutellum</i>	2	2	2.0	0	12
Ventral cirri (n)	<i>P. multinucleata</i>	44	69	58.1	6.1	25
	<i>A. scutellum</i>	–	–	–	–	–
Left marginal cirri (n)	<i>P. multinucleata</i>	60	88	76.7	6.9	25
	<i>A. scutellum</i>	11	17	13.8	1.8	12
Right marginal rows (n)	<i>P. multinucleata</i>	5	5	5.0	0	25
	<i>A. scutellum</i>	1	1	1.0	0	12
Cirri in RMR1 (n)	<i>P. multinucleata</i>	54	77	65.7	6.4	25
	<i>A. scutellum</i>	10	18	14.5	1.9	12
Cirri in RMR2 (n)	<i>P. multinucleata</i>	57	80	69.8	6.1	25
	<i>A. scutellum</i>	–	–	–	–	–
Cirri in RMR3 (n)	<i>P. multinucleata</i>	64	86	73.9	6.6	25
	<i>A. scutellum</i>	–	–	–	–	–
Cirri in RMR4 (n)	<i>P. multinucleata</i>	69	94	78.4	7.4	25
	<i>A. scutellum</i>	–	–	–	–	–
Cirri in RMR5 (n)	<i>P. multinucleata</i>	67	94	80.7	6.9	25
	<i>A. scutellum</i>	–	–	–	–	–
Macronuclear nodules (n)	<i>P. multinucleata</i>	43	70	54.8	8.0	25
	<i>A. scutellum</i>	42	90	61.7	15.0	12
Micronuclei (n)	<i>P. multinucleata</i>	6	12	10.0	2.0	12
	<i>A. scutellum</i>	2	2	2.0	0	12
Dorsal kineties (n)	<i>P. multinucleata</i>	3	3	3.0	0	25
	<i>A. scutellum</i>	3	3	3.0	0	12

ventral cirri (44–69 vs 27–45), membranelles (52.4 vs 46.7 on average) and left marginal cirri (76.7 vs 61.3 on average) (Tables 1 and 2; Figs 1a, f and 4a, b). In addition, *P. multinucleata* has two types of cortical granules, which are possibly absent in *P. similis*. The dissimilarity between these two forms is also supported by the molecular data, their SSU rRNA gene sequences differing by 21 substitutions and 2 indels (i.e. 1.3 %) (Fig. 3b). These findings support the

validity of *P. multinucleata* as a novel species (Hu *et al.*, 2002).

In terms of its morphology *in vivo* (e.g. body shape and size, number of macronuclear nodules, cell colour, etc.) and the basic pattern of its infraciliature (i.e. one left marginal row and more than one right marginal row), *P. multinucleata* resembles two other morphotypes, *Birojimia muscorum*

Table 2. Morphological comparison of *P. multinucleata* spec. nov. with *P. similis* and some closely related morphotypes of *Birojimia*

Data were taken from this study (*P. multinucleata*), Hu *et al.* (2002) (*P. similis*), Berger & Foissner (1989) (*B. terricola*) and Foissner (1982) (*B. muscorum*).

Character	<i>P. multinucleata</i>	<i>P. similis</i>	<i>B. terricola</i>	<i>B. muscorum</i>
Body length <i>in vivo</i> (µm)	150–350	140–300	135–235	150–250
Cortical granules	Two types, colourless	Absent	Absent	Colourless
Adoral membranelles (<i>n</i>)	46–60 (<i>n</i> =25)	40–53 (<i>n</i> =23)	35–43 (<i>n</i> =12)	26–32 (<i>n</i> =10)
Buccal cirri (<i>n</i>)	1 (<i>n</i> =25)	1 (<i>n</i> =25)	1 (<i>n</i> =12)	1 (<i>n</i> =10)
Frontal cirri (<i>n</i>)	3 (<i>n</i> =25)	3 (<i>n</i> =25)	3 (<i>n</i> =12)	3 (<i>n</i> =10)
Frontoterminal cirri (<i>n</i>)	Lacking	Lacking	2 (<i>n</i> =12)	Lacking
Midventral cirri (<i>n</i>)	6–16 (<i>n</i> =25)	7–12 (<i>n</i> =21)	26 (<i>n</i> =12)	21–33 (<i>n</i> =10)
Transverse cirri (<i>n</i>)	6–11 (<i>n</i> =25)	3–11 (<i>n</i> =21)	4–6 (<i>n</i> =12)	2–4 (<i>n</i> =10)
Ventral cirri (<i>n</i>)	44–69 (<i>n</i> =25)	27–45 (<i>n</i> =21)	Lacking	7
Left marginal cirri (<i>n</i>)	60–88 (<i>n</i> =25)	50–79 (<i>n</i> =21)	40–53 (<i>n</i> =12)	34–52 (<i>n</i> =10)
Right marginal rows (<i>n</i>)	5 (<i>n</i> =25)	5–8 (<i>n</i> =21)	2–6 (<i>n</i> =12)	2 (<i>n</i> =10)
Caudal cirri (<i>n</i>)	Absent	Absent	2–7 (<i>n</i> =12)	4–7 (<i>n</i> =10)
Macronuclear nodules (<i>n</i>)	43–70 (<i>n</i> =25)	3–6 (<i>n</i> =24)	55–65 (<i>n</i> =12)	20–52 (<i>n</i> =10)
Dorsal kinetics (<i>n</i>)	3 (<i>n</i> =25)	3 (<i>n</i> =25)	5 (<i>n</i> =12)	4 (<i>n</i> =10)
Habitat	Marine	Marine	Soil	Soil

(Kahl, 1932) Berger & Foissner, 1989 and *Birojimia terricola* Berger & Foissner, 1989. However, *B. muscorum* differs from *P. multinucleata* in habitat (soil vs marine), possession of caudal cirri (absent in *P. multinucleata*), having more

midventral pairs (approx. 11 vs 5), midventral row composed of fewer cirri (approx. 7 vs approx. 55) and in having fewer membranelles (26–32 vs 46–60), right marginal rows (2 vs 5) and transverse cirri (2–4 vs 6–11) (Table 2)

(Kahl, 1932; Berger & Foissner, 1989; Berger, 2006). Likewise, *P. multinucleata* differs from *B. terricola*, which usually has two to six RMR (invariably five in *P. multinucleata*), two frontoterminal cirri (frontoterminal cirri absent), two to seven caudal cirri (caudal cirri absent), no midventral row (midventral row composed of 44–69 cirri), more midventral pairs (approx. 13 vs approx. 5) and inhabits soil (marine) (Table 2) (Foissner, 1982; Berger & Foissner, 1989).

***Anteholosticha scutellum* (Cohn, 1866) Berger, 2003 (Fig. 5 and Tables 1 and 3)**

Syn. The detailed revision was listed by Berger (2006).

No investigations have been conducted for this species using modern methods. We therefore provide an improved diagnosis based on our observations.

Improved diagnosis. Small, oval *Anteholosticha*, about 50–80 × 20–30 µm *in vivo*; about 17 adoral membranelles; three frontal, one buccal, two frontoterminal, approx. eight transverse and two pretransverse cirri; midventral complex extends approx. 60% of cell length and comprises six or seven cirral pairs; 10–18 right and 11–17 left marginal cirri; three dorsal kineties; about 60 macronuclear nodules; cortical granules large and scattered over dorsal side of body; marine habitat.

Slide deposition. Since no type specimens of *Anteholosticha scutellum* are known to exist, voucher slides of protargol-impregnated cells have been deposited in the collections of the Laboratory of Protozoology, OUC, China (registration number 2008:6:1:2) and the Natural History Museum, London, UK (registration number 2008:5:13:2).

Description. Cell highly flexible, slightly contractile when stimulated, approx. 50–75 × 20–30 µm *in vivo*, outline shape usually ellipsoid, length-to-width ratio about 2–2.5:1 (Fig. 5a, f–h), dorsoventrally flattened about 3:1 (Fig. 5b, k). Buccal field about 25–35% of cell length.

Cortical granules (possibly extrusomes) spherical and colourless, approx. 1 µm across, irregularly scattered on dorsal side (Fig. 5c, o). In protargol-stained specimens, 2–3 µm-long thorns were observed, having been ejected from these granules (Fig. 5e, m, n).

Cytoplasm colourless to slightly greyish, with several granules (<2 µm) and numerous food vacuoles (approx. 5 µm in diameter). About 60 ellipsoid macronuclear nodules, difficult to observe *in vivo* (Fig. 5e, l, m). Two Mi recognizable in some specimens (Fig. 5e, m).

Locomotion by crawling, medium-fast on substratum, with no peculiarities.

Infraciliature as shown in Fig. 5d. AZM evenly curved, with 17 or 18 membranelles, cilia of membranelles about 8 µm long. Undulating membranes almost parallel, straight or

slightly curved (Fig. 5d, l). Three enlarged frontal cirri (FC; Fig. 5d). One buccal cirrus near anterior end of undulating membranes (BC; Fig. 5d). Two frontoterminal cirri (FTC; Fig. 5d) between distal end of AZM and anterior end of RMR (Fig. 5d). Midventral complex is composed of six or seven pairs of cirri and terminates at about the equatorial level of the cell (MVC; Fig. 5d). Eight strong transverse cirri (TC; Fig. 5d) arranged in U-shaped row; always two pretransverse ventral cirri (PTC; Fig. 5d) between transverse cirri and midventral rows. Both left and right marginal rows consist of approx. 15 cirri (Fig. 5d). DK extend whole length of body; dorsal cilia sparsely distributed, about 3 µm long *in vivo* (Fig. 5e).

SSU rRNA gene sequence (Fig. 3a, c and Table 4). The GenBank/EMBL/DDBJ accession number for the SSU rRNA gene sequence of *A. scutellum* is FJ156105 (1773 bp). Regarding sequence similarity among *Anteholosticha* species, *A. scutellum* differs from *A. manca* by 147 nucleotides, from *Anteholosticha* sp1-QD-07060701 by 111 nucleotides, from *A. multistilata* by 92 nucleotides and from *Anteholosticha* sp2-QD-04082401 by 46 nucleotides (Fig. 3a). In the phylogenetic trees, *A. scutellum* forms a strongly supported clade with *Anteholosticha* sp2-QD-04082401 and *Holosticha heterofoissneri*. It should be noted that the genus *Anteholosticha* is not monophyletic: its congeners are distributed in four clades (Fig. 3c).

Remarks and comparison

Anteholosticha scutellum was first described as *Oxytricha scutellum* Cohn, 1866. The original description does not include information about the cirral pattern or nuclear apparatus because, when viewed *in vivo*, cells are mostly opaque as a result of the blackish granules and many other cytoplasmic inclusions (Fig. 4c, d).

Kahl (1932, 1933) provided a short but significant characterization of this species (which he called *Holosticha scutellum*), thus: 60–120 µm long *in vivo*, body outline usually oval, many macronuclear nodules, two conspicuous micronuclei, three slightly enlarged frontal cirri, midventral complex composed of cirral pairs, two pretransverse ventral cirri and seven or eight transverse cirri, caudal cirri absent (Fig. 4e, f). Berger (2003) redefined the genus *Holosticha* and transferred this species to the genus *Anteholosticha*, which includes forms with a continuous AZM that lack caudal cirri. In terms of living features, the organism we isolated corresponds perfectly with the descriptions of Cohn (1866) and (Kahl 1932, 1933); therefore, its identity is not in doubt.

Entz (1884) illustrated and described a form that he called *Holosticha scutellum* which had two ellipsoidal macronuclear nodules, each with an accompanying micronucleus, seven to 11 extremely strong transverse cirri arranged in a crescent-shaped row, two ventral cirral rows that extend from the frontal region nearly to the right transverse

Fig. 4. Morphology of some previously reported nominal species. (a, b) *P. similis* (from Hu *et al.*, 2002). (c–i) *A. scutellum* [c, d from Cohn (1866); e from Kahl (1933); f from Kahl (1932); g from Mansfeld (1923); h from Entz (1884); i from Borror & Wicklow (1983)]. (j–l) *A. warreni* (from Song & Wilbert, 1997a). (m–o) *A. manca* (from Song & Wilbert, 1997b). (p, q) *Holosticha* sp. (from Wilbert & Song, 2005). Bars, 60 μ m (a, b), 40 μ m (j–l, p, q) and 50 μ m (m–o).

cirri and one contractile vacuole near the left cell margin in the equatorial region of the cell (Fig. 4h). As noted by Berger (2006), this form is very similar to *Holosticha diademata*, so it is likely that Entz (1884) actually observed

a population of this latter species rather than *A. scutellum*. Mansfeld (1923) also described a form under the name *Holosticha scutellum* with numerous macronuclear nodules and eight to ten transverse cirri arranged in a J-shaped row.

Fig. 5. *Anteholosticha scutellum* observed *in vivo* (a–c, f–k, o) and after protargol impregnation (d, e, l–n). (a) Ventral view of a typical individual. (b, k) Lateral views. (c) Dorsal view, showing the spherical cortical granules irregularly arranged. (d, e, l, m) Ventral (d, l, m) and dorsal (e) views showing the infraciliature; arrowheads in (e) depict the micronuclei. (f–j) Variations in body shape. (n) Dorsal view to show the dorsal kineties (arrowheads) and extrusomes (arrow). (o) Dorsal view to show the irregularly arranged spherical cortical granules. Ex, Extrusome; FTC, frontoterminal cirri; PTC, pretransverse ventral cirri; TC, transverse cirri. See Fig. 1 for other definitions. Bar, 25 μ m.

However, according to Mansfield's illustration, this organism has a wider AZM and more marginal cirri and midventral cirral pairs (Fig. 4g) than our population or that of Kahl (1932, 1933). We therefore conclude that Mansfield (1923) observed a different form and not *A. scutellum*.

In terms of body size, numbers of macronuclear nodules, basic patterns of infraciliature and marine habitat, two congeners should be compared with *A. scutellum*: *Anteolosticha warreni* and *A. manca* (Song & Wilbert, 1997a, b; Berger, 2003). Both are characterized by having a continuous AZM, a midventral row in a typical zigzag pattern, distinctly enlarged frontal cirri and no caudal cirri. However, *A. warreni* differs from *A. scutellum* in having more adoral membranelles (26–31 vs 17–18), midventral cirri (7–9 vs 6–7), transverse cirri (approx. 11 vs approx. 8), left marginal cirri (22–27 vs approx. 14), and right marginal cirri (21–26 vs approx. 15). In addition, *A. warreni* has cortical granules which are approx. 2 µm in size (vs approx. 1 µm), ellipsoid and flattened with a central depression (spherical), and arranged dorsally in three loosely organized rows (scattered randomly over the dorsal side) (Table 3; Fig. 4j–l) (Song & Wilbert, 1997a). Borror & Wicklow (1983) made a detailed redescription of a form they called *A. scutellum*. The specimen they illustrated, however, has about 34 adoral membranelles and about 30 right and 34 left marginal cirri (Fig. 4i). It is therefore likely that Borror & Wicklow (1983) had observed a population of *A. warreni* rather than *A. scutellum*.

A. manca can be distinguished from *A. scutellum* in its body shape (fusiform vs elliptical), the arrangement of

cortical granules (irregularly aligned in rows vs scattered randomly) and numbers of adoral membranelles, frontal cirri, frontoterminal cirri, midventral cirri, transverse cirri, left marginal cirri and right marginal cirri (Table 3; Fig. 4m–o) (Song & Wilbert, 1997b).

Anteolosticha arenicola (Kahl, 1932) resembles *A. scutellum* in body size and shape, marine habitat and some morphometric characters (e.g. numbers of membranelles, frontal cirri, transverse cirri, left marginal cirri and right marginal cirri). However, the former can be clearly separated from the latter by having two macronuclear nodules (vs 42–90 in *A. scutellum*) (Table 3) (Kahl, 1932).

Wilbert & Song (2005) described *Holosticha* sp. from King George Island, Antarctica. Unfortunately, only a small number of protargol-impregnated individuals were observed, and no data were collected from live observations. Nevertheless, it is likely that this form represents a population of *A. scutellum*, since it has the same numbers of membranelles, frontal cirri, frontoterminal cirri, buccal cirri and left marginal cirri and similar numbers of midventral cirri (3 or 4 vs 6 or 7), right marginal cirri (17 vs 15), dorsal kineties (4 vs 3) and macronuclear nodules (approx. 30 vs approx. 60) (Table 3; Fig. 4p–q) (Berger, 2006; Wilbert & Song, 2005).

Molecular comparison with congeners

The separation of *A. scutellum* from *A. manca*, *Anteolosticha* sp1 QD-07060701, *Anteolosticha* sp1 QD-04082401 and *A. multistilata* is supported by differences in their SSU rRNA gene sequences and its unambiguous

Table 3. Morphological comparison of *Anteolosticha scutellum* with some related species

Data were taken from this study (*A. scutellum*), Song & Wilbert (1997a) (*A. warreni*), Song & Wilbert (1997b) (*A. manca*), Kahl (1932) (*A. arenicola*) and Wilbert & Song (2005) (*Holosticha* sp.). NA, Data not available. All species are marine.

Character	<i>A. scutellum</i>	<i>A. warreni</i>	<i>A. manca</i>	<i>A. arenicola</i>	<i>Holosticha</i> sp.
Body length <i>in vivo</i> (µm)	50–80	80–120	76*	70–90	65*
Body shape	Elliptical, both ends rounded	Elliptical, both ends narrowly rounded	Fusiform, both ends rounded	Symmetrical ovoid	NA
Cortical granules	Scattered, ~1 µm	Erythrocyte in shape, ~2 µm	Irregularly aligned in rows, ~1 µm	NA	NA
Adoral membranelles (<i>n</i>)	17–18 (<i>n</i> =12)	26–31 (<i>n</i> =16)	21–27 (<i>n</i> =11)	23†	~17
Buccal cirri (<i>n</i>)	1 (<i>n</i> =12)	1 (<i>n</i> =30)	1 (<i>n</i> =16)	NA	1
Frontal cirri (<i>n</i>)	3 (<i>n</i> =12)	3 (<i>n</i> =30)	4 (<i>n</i> =16)	3	3
Frontoterminal cirri (<i>n</i>)	2 (<i>n</i> =12)	2 (<i>n</i> =30)	2–4 (<i>n</i> =16)	NA	2
Midventral cirrus pairs (<i>n</i>)	6–7 (<i>n</i> =12)	7–9 (<i>n</i> =11)	9–16 (<i>n</i> =11)	10†	~3 or 4
Transverse cirri (<i>n</i>)	~8 (<i>n</i> =12)	~11 (<i>n</i> =16)	4–6 (<i>n</i> =16)	7–10†	6–7
Left marginal cirri (<i>n</i>)	~14 (<i>n</i> =12)	22–27 (<i>n</i> =16)	22–29 (<i>n</i> =11)	11†	14
Right marginal cirri (<i>n</i>)	~15 (<i>n</i> =12)	21–26 (<i>n</i> =16)	6–34 (<i>n</i> =11)	13†	17
Macronuclear nodules (<i>n</i>)	42–90 (<i>n</i> =12)	~50	~50–70	2	~30
Dorsal kineties (<i>n</i>)	3 (<i>n</i> =12)	3 (<i>n</i> =16)	3 (<i>n</i> =16)	NA	4

*Data from protargol-impregnated specimens.

†Determined from illustration.

Table 4. SSU rRNA gene sequence similarity (%) among *A. scutellum* and four congeners

Species	1	2	3	4	5
1. <i>A. scutellum</i>	(100)				
2. <i>A. manca</i>	91.4	(100)			
3. <i>Anteholosticha</i> sp1-QD-04082401	93.4	94.1	(100)		
4. <i>Anteholosticha</i> sp2-QD-07060701	97.3	90.7	90.9	(100)	
5. <i>A. multistilata</i>	94.5	91.2	95.6	91.2	(100)

position in the phylogenetic trees (Fig. 3a, c). Pairwise sequence similarities between *A. scutellum* and its congeners range from 91.4 to 97.3% (Table 4), i.e. the differences range from 2.7 to 8.6%. Such molecular differences, combined with its unique morphological and morphogenetic features, support the conclusion that *A. scutellum* is a well-outlined and distinctive member of the genus *Anteholosticha*.

Berger (2003) split *Anteholosticha* from *Holosticha* based on the absence of several *Holosticha* apomorphies and caudal cirri, and also predicted the heterogeneity of *Anteholosticha*. In the present and previous studies, SSU rRNA gene trees have supported the clear separation of *Holosticha* and *Anteholosticha*. Meanwhile, consistent with the hypothesis of Berger (2003) and previous molecular studies (Schmidt *et al.*, 2007; Yi *et al.*, 2008), *Anteholosticha* is recovered as a conspicuously divergent group, with its congeners distributed in four separate clades.

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