Taxonomy and phylogeny of *Tunicothrix* (Ciliophora, Stichotrichia), with the description of two novel species, *Tunicothrix brachysticha* n. sp. and *Tunicothrix multinucleata* n. sp., and the establishment of Parabirojimidae n. fam.

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The morphology and molecular phylogeny of two novel urostylid ciliates, *Tunicothrix brachysticha* n. sp. and *Tunicothrix multinucleata* n. sp., were investigated using the techniques of living observation, protargol impregnation and small-subunit rRNA gene sequencing. Both species were found to be frequent in muddy-sand sediment but very rare in sandy sediment of the intertidal zone of Qingdao Bay, China. *Tunicothrix brachysticha* is about $90 \times 30 \mu\text{m}$ *in vivo*, with a broadly clavate body shape, and is characterized by a rather short midventral row terminating at 40 % of the body length and a right marginal row 1 terminating at mid-body. *Tunicothrix multinucleata* is about $190 \times 30 \mu\text{m}$ *in vivo*, and is characterized by a clavate body shape and the presence of 7–10 macronuclear nodules. Phylogenetic analyses showed that *Tunicothrix* clustered robustly with *Parabirojimia*, forming a clade that branches from the Urostylida core clade with high bootstrap values. Based on the morphological and molecular data, we establish a new family, Parabirojimidae n. fam., which comprises *Parabirojimia* and *Tunicothrix* and is distinguished by a unique, roughly T-shaped bipartite adoral zone of membranelles, three enlarged frontal cirri and a midventral complex with midventral pairs confined to the buccal field.

INTRODUCTION

The ciliate subclass Stichotrichia *sensu* Lynn & Small (2002) and Lynn (2008) comprises 13 families and is an especially difficult group in its systematics and phylogeny (e.g. Foissner *et al.*, 2004; Liu *et al.*, 2010; Schmidt *et al.*, 2007; Yi *et al.*, 2008). In spite of many studies focusing on this group, it is still difficult to reconcile the morphological classification and molecular phylogeny of Stichotrichia, probably because of parallel evolution and undersampling of taxa (Foissner *et al.*, 2004). The genus *Tunicothrix* Xu *et al.*, 2006 is a small group of urostylids characterized by a conspicuous cortical alveolar layer (AL), a bipartite adoral zone of membranelles (AZM) and a few midventral pairs confined to the oral region. In the absence of molecular phylogeny data, *Tunicothrix* was originally assigned to the family Urostylidae based on the presence of midventral

pairs (Xu *et al.*, 2006). So far, only two species have been described: the type species *Tunicothrix rostrata* Xu *et al.*, 2006 and *Tunicothrix wilberti* (Lin & Song, 2004). In this paper, we describe two novel species of *Tunicothrix* isolated from intertidal sediment of Qingdao Bay, China, and try to estimate the phylogenetic position of the genus based on protargol-impregnated specimens and small-subunit (SSU) rRNA gene sequence analysis.

METHODS

Sample collection and identification. The two species were isolated from muddy-sand sediment (55.7 % sand, 36.6 % silt, 7.7 % clay) of the intertidal zone of Qingdao Bay, China ($36^{\circ} 03' \text{ N } 120^{\circ} 19' \text{ E}$). Samples used for morphological study were collected in May 2008 and May 2010, with interstitial water temperature about 13°C and salinity about 29 ‰. In addition, *Tunicothrix brachysticha* n. sp. was also collected in March 2009 for molecular investigation. Living cells were isolated and observed with differential interference contrast microscopy and impregnated with the protargol method of Wilbert (1975) and the quantitative protargol stain (QPS) following the modification of Skibbe (1994). Drawings of living cells were based on free-hand sketches; those of stained cells were made with the help of a drawing device. Terminology is mainly according to Xu *et al.* (2006).

Abbreviations: AL, cortical alveolar layer; AZM, adoral zone of membranelles; BI, Bayesian inference; ML, maximum-likelihood; NJ, neighbour-joining; QPS, quantitative protargol stain; SSU, small subunit.

The GenBank/EMBL/DDBJ accession number for the SSU rRNA gene sequence of *Tunicothrix brachysticha* n. sp. isolate QD-20080519-01 is GU574811.

DNA extraction and sequencing. The REDExtract-N-Amp Tissue PCR kit (Sigma) was applied for DNA extraction. Cells of *T. brachysticha* were isolated and washed about three times using autoclaved seawater to remove other protists; a single cell was then picked out with a micropipette and processed according to the manufacturer's protocol. SSU rRNA gene amplification [forward primer Euk A (5'-AACCTGGTTGATCCTGCCAGT-3'), reverse primer Euk B (5'-TGATCCTTCTGCAGGTCACCTAC-3'); Medlin *et al.*, 1988] was performed according to Yi *et al.* (2008). Purified PCR products were inserted into the pUCm-T vector (Sangon) and sequenced at the Invitrogen sequencing facility (Shanghai, China).

Sequence availability and phylogenetic analyses. All sequences used for phylogenetic analyses were obtained from GenBank: a total of 61 sequences from six subclasses of Spirotrichea (*sensu* Lynn, 2008) (Licnophoria was not included) were included in the phylogenetic analyses; *Blepharisma americanum* was taken as an outgroup taxon. Sequence alignments were performed using CLUSTAL W (Thompson *et al.*, 1994) implemented in BioEdit 7.0.0 (Hall, 1999) with default parameters. Neighbour-joining (NJ) trees were constructed with the PHYLIP package version 3.68 (Felsenstein, 2005). Evolutionary distances were calculated with Kimura's two-parameter model, using a transition/transversion ratio of 2.0. Maximum-likelihood (ML) analyses were performed with PhyML version 2.4.4 (Guindon & Gascuel, 2003) using the evolutionary model GTR+I+G selected by Modeltest 3.7 (Posada & Crandall, 1998) with I=0.4342 and G=0.4754. Using the same model, Bayesian inference (BI) analysis was conducted with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) with 1 000 000 generations and an initial burn-in of 2500. Phylogenetic trees were viewed with TreeView version 1.6.6 (Page, 1996) and MEGA 4.0 (Tamura *et al.*, 2007).

RESULTS

Tunicothrix brachysticha n. sp. (Figs 1a–d and 2; Table 1)

Diagnosis. Size about $90 \times 30 \mu\text{m}$ *in vivo*; body broadly clavate with an inconspicuous beak-like protrusion at subapical-left corner; two macronuclear nodules and several micronuclei; 6–9 frontal and 14–17 ventral membranelles; three frontal, one buccal, usually one pretransverse and three transverse cirri; two or three midventral pairs followed by a midventral row composed of 8–13 cirri terminating at about 40 % of body length; 17–30 cirri in left marginal row, 16–22 cirri in right marginal row 1 terminating at mid-body and 23–35 cirri in right marginal row 2; three dorsal kineties.

Type locality. Intertidal sediment of Qingdao Bay, China ($36^{\circ} 03' \text{N}$ $120^{\circ} 19' \text{E}$).

Type specimens. The holotype slide (no. QD-20080519-01) with protargol-impregnated specimens has been deposited in the Marine Biological Museum, Chinese Academy of Sciences, Qingdao; one paratype slide from the same series has been deposited in the Natural History Museum, London, UK, with accession number 2010:6:22:1. The holotype and paratype specimens are marked with black-ink circles on the cover glass.

Etymology. Composite of the Greek words *brachy* (short) and *stichus* (in a row), referring to the shortened midventral row and right marginal row 1 of the species.

Morphological description. Size *in vivo* $60\text{--}110 \times 25\text{--}40 \mu\text{m}$, usually about $90 \times 30 \mu\text{m}$; length-to-width ratio near 3:1 *in vivo* and in silver-impregnated specimens (Table 1). Body broadly clavate with an inconspicuous beak-like protrusion at subapical-left corner, where it segregates the frontal and ventral membranelles (Figs 1a and 2b); widest at anterior one-quarter to one-third body portion (Figs 1a, b and 2a–c). Invariably two macronuclear nodules lie in middle body portion with a fine thread frequently recognizable between them; individual nodules ovoid to elliptical, about $13 \times 6 \mu\text{m}$. Two to four globular micronuclei near or attached to macronuclear nodules (Figs 1d and 2h). No contractile vacuole observed. AL very conspicuous, almost hyaline and $3\text{--}5 \mu\text{m}$ thick *in vivo*, also detectable in well-impregnated specimens (Figs 1a, b and 2a–c). Cells neither flexible nor contractile, but very fragile and easily broken when irritated. Cytoplasm invariably contains some colourless, arc-shaped structures, $10\text{--}15 \times 0.8\text{--}1 \mu\text{m}$, near dorsal side of cell and many body inclusions, $2\text{--}5 \mu\text{m}$ across, over the whole body (Figs 1a, b and 2b, c). The arc-shaped structures remain intact and refractive but motionless when cell is ruptured; we could not decide whether these belong to an endosymbiont or are structures of the cell. Movement relatively fast; cells prefer to move on the bottom or substrate particles in Petri dish culture, sometimes rotate around sediment particles.

AZM distinctly bipartite, forming a roughly T-shaped pattern; composed of frontal and ventral membranelles occupying 28 % of body length on average (Table 1; Figs 1a, c, d and 2b–d). Frontal portion of adoral zone composed of 6–9 membranelles with cilia about $13 \mu\text{m}$ long and ventral portion of adoral zone composed of 14–17 membranelles. Behind frontal membranelles, many small rods impregnated with protargol appearing as a row of minute cirri, but bearing no cilia. Three enlarged frontal cirri behind the row of small rods. One buccal cirrus lies at right of parallel paroral and endoral membranes. Midventral complex composed of 2–3 cirral pairs in zigzag pattern (Figs 1c, d and 2d, f, h) and a midventral row consists of 7–10 cirri and terminates on average at 40 % of body length (Figs 1c, d and 2h). Three transverse cirri with cilia about $15 \mu\text{m}$ long located in rear body end; usually one pretransverse cirrus lies ahead of transverse cirri (Figs 1a, d and 2e). Left marginal row composed of 17–30 cirri extending from the middle left of ventral membranelle to a subterminal body position; anteriormost cirrus has different polarization from other cirri (Figs 1c, d and 2g). Right marginal row 1 composed of 16–22 cirri terminating in mid-body; right marginal row 2 composed of 23–35 cirri extending to rear body end (Figs 1a, d and 2d, e). Three dorsal kineties extend to entire body length.

Fig. 1. *Tunicothrix brachysticha* n. sp. (a–d), *T. multinucleata* n. sp. (e–g), *T. wilberti* (h, i) [from Lin & Song (2004)] and *T. rostrata* (j, k) [from Xu *et al.* (2006)], from life (a, b, e, h, j) and after QPS staining (c, d, f, g) and Wilbert's protargol method (i, k). (a) Ventral view of representative specimen. Arrow denotes the beak-like protrusion at anterior corner. (b) Dorsal view showing arc-shaped structure (arrow). (c) Ventral cirral pattern of anterior body portion. (d) Infraciliature and nuclear pattern in ventral view of holotype specimen. Arrow marks the anteriormost cirrus, which is separated from the main row by different polarization. (e) Ventral view of representative specimen. (f) Ventral view of anterior body portion. (g) Infraciliature and nuclear pattern in ventral view of holotype specimen. *T. multinucleata* differs from congeners by having 7–10 macronuclear nodules. (h, i) Ventral views of *T. wilberti*. The midventral row (arrow) and right marginal row 1 (arrowhead) are distinctly longer than those in *T. brachysticha* (d). (j, k) Ventral and dorsal views of *T. rostrata*. AL, Cortical alveolar layer; BC, buccal cirrus; E, endoral; FC, frontal cirri; FM, frontal membranelles; LMR, left marginal row; MA, macronucleus; MI, micronucleus; MP, midventral pairs; MV, midventral row; P, paroral; PT, pretransverse cirrus; RMR1, right marginal row 1; RMR2, right marginal row 2; TC, transverse cirri; VM, ventral membranelles. Bars, 30 µm.

SSU rRNA gene sequence. The SSU rRNA gene sequence of *T. brachysticha* is deposited in the GenBank database with the accession number GU574811 and length of 1779 bp.

Occurrence and ecology. We found *T. brachysticha* in each month except August–October during monthly

samplings in the intertidal area of Qingdao Bay from July 2007 to June 2008. This ciliate was frequent in muddy-sand sediment but very rare in sandy sediment (96.9 % sand, 2.4 % silt, 0.8 % clay) of the intertidal zone. It was mostly distributed in the top 2 cm of the muddy-sand sediment, especially in the 0.5 cm surface layer, where it occurred at

Fig. 2. *Tunicothrix brachysticha* n. sp. from life (a–c) and after QPS staining (d–h). (a) Ventral view of a slender specimen. (b, c) Ventral and dorsal views of the same specimen, slightly squashed. Arrows denote the beak-like protrusion (b) and an arc-shaped structure (c). (d–h) Ventral views of four specimens showing the ventral cirral pattern. Arrow in (g) marks the anteriormost cirrus clearly separated from the main row. See legend to Fig. 1 for abbreviations. Bars, 30 µm.

high abundance in April to June and peaked at about 700 cells cm^{-3} in May 2008 (with interstitial water temperature 13 °C and salinity about 29‰). *T. brachysticha* is probably an algivorous species, because we often observed undigested prey diatoms in the food vacuoles of the cells, and the ciliate could not survive or grow on bacteria.

***Tunicothrix multinucleata* n. sp. (Figs 1e–g and 3; Table 1)**

Diagnosis. Size about 190×30 µm *in vivo*; body clavate with a beak-like protrusion at subapical-left corner; 7–10 (usually 8) macronuclear nodules and several micronuclei; 7–10 frontal and 13–19 ventral membranelles; three frontal, one buccal, one pretransverse and four or five transverse

cirri; three or four midventral pairs followed by a midventral row of 8–11 cirri terminating at anterior fourth of body; 38–53 cirri in left marginal row, 20–27 cirri in right marginal row 1 terminating at anterior third of body, with 44–61 cirri in right marginal row 2; three dorsal kineties.

Type locality. Intertidal sediment of Qingdao Bay, China (36° 03' N 120° 19' E).

Type specimens. The holotype slide (no. QD-20080519-03) with protargol-impregnated specimens has been deposited in the Marine Biological Museum, Chinese Academy of Sciences, Qingdao; one paratype slide from the same series has been deposited in the Natural History

Table 1. Morphometric data for *T. brachysticha* n. sp. (first row), *T. multinucleata* n. sp. (second row), *T. rostrata* (third row) and *T. wilberti* (fourth row)

Min., Minimum; Max., maximum; Mean, arithmetic mean; SD, standard deviation; CV, coefficient of variation (%); *n*, number of individuals analysed. Data for *T. rostrata* and *T. wilberti* were taken from Xu *et al.* (2006) and Lin & Song (2004), respectively, unless indicated. Data were based on Wilbert's protargol method unless indicated. –, No data available.

Characteristic	Min.	Max.	Mean	Median	SD	CV	<i>n</i>
Body length (µm)	55.0	93.5	77.0	78.0	9.1	11.8	21
	110.0	214.0	162.3	160.0	28.7	17.7	15*
	125.0	185.0	156.4	155.0	19.7	12.6	21
	92.0	160.0	121.1	124.0	21.4	17.6	16
Body width (µm)	20.3	42.0	27.3	27.0	5.4	19.8	21
	21.0	40.0	28.7	28.0	4.7	16.5	15*
	35.0	80.0	62.1	63.0	13.6	21.9	21
	28.0	60.0	42.9	44.0	10.0	23.4	16
Body length-to-width ratio	1.6	4.5	2.9	2.8	0.6	22.1	21
	3.4	8.2	5.8	5.4	1.4	24.5	15*
	1.6	4.3	2.6	2.7	0.7	24.9	21
AZM length (µm)	–	–	–	–	–	–	–
	16.0	24.5	20.6	21.0	2.0	9.8	20
	24.0	29.0	26.5	26.0	1.7	6.4	15*
	34.0	55.0	44.8	45.0	5.8	13.0	21
Adoral zone length-to-body length ratio	20.0	32.0	26.8	27.0	3.3	12.3	16
	0.23	0.33	0.28	0.29	0.03	10.60	21
	0.12	0.21	0.16	0.15	0.03	16.25	21*
Macronuclei, number	0.2	0.4	0.3	0.3	0.1	18.3	21
	–	–	–	–	–	–	–
	2	2	2.0	2.0	0.0	0.0	21
	7	10	8.2	8.0	0.7	8.4	19*
Micronuclei, number	2	2	2.0	2.0	0.0	0.0	21
	2	2	2.0	2.0	0.0	0.0	50
	2	4	2.4	2.0	0.7	31.1	21
	3	5	3.7	3.5	0.8	22.3	6*
Frontal adoral membranelles, number	2	3	2.1	2.0	0.3	14.4	21
	1	3	2.0	2.0	0.4	20.4	13
	6	9	7.4	7.0	0.7	10.0	21
	7	10	8.8	9.0	0.7	8.3	13*
Ventral adoral membranelles, number	14	18	15.4	15.0	1.5	9.5	21
	8	12	9.8	10.0	1.0	10.0	16
	14	17	14.6	14.0	0.8	5.8	18
	13	19	17.6	18.0	1.6	8.8	13*
Frontal cirri, number	23	28	25.4	25.0	1.6	6.2	21
	16	20	18.9	20.0	1.5	7.7	16
	3	3	3.0	3.0	0.0	0.0	13
	3	3	3.0	3.0	0.0	0.0	9*
Midventral pairs, number of cirri	3	3	3.0	3.0	0.0	0.0	21
	4†	6†	5.3	4.0	1.0	19.1	16
	4	6	5.9	6.0	0.5	9.1	14
	6	8	7.0	7.0	1.1	15.3	8*
Midventral row, number of cirri	6	8	6.3	6.0	0.7	11.4	21
	–	–	–	–	–	–	–
	8	13	9.4	9.0	1.5	16.3	21*
	8	11	9.0	9.0	1.2	13.1	11*
Midventral row length-to-body length ratio	18	28	23.3	24.0	2.9	12.7	14
	20	36	25.2	25.0	3.9	15.6	16
	0.32	0.47	0.39	0.39	0.05	12.03	19*
	0.18	0.31	0.25	0.25	0.04	15.72	19*
	–	–	0.61‡	–	–	–	–
	–	–	0.60‡	–	–	–	–

Table 1. cont.

Characteristic	Min.	Max.	Mean	Median	SD	CV	n
Left marginal cirri, number	17	30	22.7	23.0	3.0	13.2	20*
	38	53	44.8	45.0	4.7	10.5	15*
	40	51	43.5	42.0	3.4	7.7	17
	21	36	29.4	31.0	3.9	13.4	16
Right marginal row 1, number of cirri	16	22	19.1	19.0	1.6	8.4	18*
	20	27	22.8	22.5	2.1	9.1	12*
	39	50	44.7	45.0	3.0	6.7	14
	31	47	35.1	37.0	4.0	11.4	16
Right marginal row 2, number of cirri	23	35	27.8	27.0	3.2	11.6	13*
	44	61	51.6	50.0	5.5	10.6	13*
	53	66	58.8	58.0	3.4	5.8	16
	18	39	31.9	31.0	5.2	16.4	16
Pretransverse ventral cirri, number	1	2	1.1	1.0	0.3	24.9	14*
	1	1	1.0	1.0	0.0	0.0	13*
	0	1	0.9	1.0	0.4	41.8	21
	0	2	1.3	1.0	0.6	46.2	16
Transverse cirri, number	3	3	3.0	3.0	0.0	0.0	13*
	4	5	4.3	4.0	0.5	11.2	13*
	3	4	3.3	3.0	0.5	14.3	17
	2	5	4.3	4.0	0.9	20.2	16
Dorsal kineties, number	3	3	3.0	3.0	0.0	0.0	5
	3	3	3.0	3.0	0.0	0.0	1*
	3	3	3.0	3.0	0.0	0.0	18
	3	3	3.0	3.0	0.0	0.0	50

*Data from QPS-stained specimen(s).

†Enumeration of frontal cirri plus midventral cirri.

‡Measurement made in this study from original illustrations.

Museum, London, UK, with accession number 2010:6:22:2. The holotype and paratype specimens are marked with black-ink circles on the cover glass.

Etymology. Composite of the Latin prefix *multi-* (many) and the Modern Latin adjective *nucleatus* (with a nucleus; from the Latin *nucleus* kernel, little nut), referring to the large number of macronuclear nodules.

Morphological description. Size *in vivo* 130–250 × 25–40 µm, usually about 190 × 30 µm; length-to-width ratio near 6:1 *in vivo* and in silver-impregnated specimens (Table 1). Body clavate with a beak-like protrusion at subapical-left corner; widest at anterior one-sixth to one-quarter body portion (Figs 1e and 3a, b). Seven to ten, usually eight macronuclear nodules in a rough line: of 19 specimens analysed, one specimen had seven nodules, two had 10 and the others had eight; individual nodules about 10 × 5 µm; 3–5 micronuclei (Figs 1f, g and 3d–f). AL very conspicuous, 3–4 µm thick (Figs 1e, g and 3a–d). Cells are flexible and fragile and easily broken when irritated. Cytoplasm invariably contains some colourless and arc-shaped structures, 18–20 × 1–1.5 µm, on dorsal side of body and many body inclusions, 1–2 µm across, over the

whole body, as mentioned above (Fig. 3b, c). Movement relatively fast. Numerous rods, 2 × 0.5 µm, dispersed in cytoplasm and faintly impregnated with the protargol method used.

AZM distinctly bipartite, occupying 12–21 % of body length; frontal portion of adoral zone composed of 7–10 membranelles and ventral portion composed of 13–19 membranelles. Three frontal, one buccal, one pretransverse and four or five transverse cirri (Figs 1e–g and 3e–g). Midventral complex composed of three or four cirral pairs in distinct zigzag pattern, posteriorly followed by a midventral row of 8–9 cirri terminating at anterior quarter of cell. Left marginal row composed of 38–53 cirri extending to subterminal body position. Right marginal row 1 composed of 20–27 cirri terminating at anterior third of body; right marginal row 2 composed of 44–61 cirri extending to rear body end. Three dorsal kineties with sparse cilia.

Occurrence and ecology. *T. multinucleata* occurred concomitantly with *T. brachysticha* in the intertidal sediment of Qingdao Bay, but at very low abundance. The highest abundance recorded was about 10 cells cm^{−3} in 0–0.5 cm deep sediment. Like *T. brachysticha*, the

Fig. 3. *Tunicothrix multinucleata* n. sp. from life (a–c) and after QPS staining (d–g). (a) Ventral view of a representative specimen. (b, c) Flexible body and AL. (d) Dorsal view showing the macronuclear pattern and the conspicuous AL. (e–g) Ventral view to show the anterior (e, f) and posterior (g) region of different specimens. See legend to Fig. 1 for abbreviations. Bars, 30 µm.

species is algivorous and was mostly found from the muddy-sand sediment and very rare in the sandy sediment.

Phylogeny inferred from SSU rRNA gene sequences

Based on the dataset of 62 taxa, the BI and NJ trees show similar topologies, which were basically congruent with the ML tree presented in Fig. 4. The Protocruziidia branches at the base of this tree, followed by the Phacodiniidia and the Hypotrichia (Fig. 4). The subclasses Oligotrichia and Choreotrichia cluster together within the Stichotrichia in the ML and BI trees, but form a sister group to Stichotrichia

in the NJ tree (56%). The phylogenetic relationships of the subclass Stichotrichia *sensu* Lynn & Small (2002) are rather complicated and it is roughly separated into three groups: a Parabirojimidiae clade plus a *Trachelostyla pediculiformis* clade, a Urostylida core clade consistently composed of 16 taxa and a confusing Sporadotrichida-like group (Fig. 4). The *Holosticha* (*sensu* Berger, 2003) clade and *Psammomitra retractilis* are separated from the Urostylida core clade, but their position is not well established. *Anteholosticha multistilata* branches within the Urostylida core clade in the BI tree, but clusters with *Amphisiella magnigranulosa* and *Orthoamphisiella breviseris* within the Sporadotrichida group in the ML and NJ trees. Moreover, *Anteholosticha*

Fig. 4. Phylogenetic position of *T. brachysticha* inferred from SSU rRNA gene sequences using the ML method. GenBank accession numbers of the sequences follow the species names. Numbers at nodes represent bootstrap percentages for the ML tree, posterior probabilities for the BI tree and bootstrap percentages for the NJ tree (–, <50%). Clades with different topologies in the BI and/or NJ trees relative to the ML tree are marked with asterisks. Bar, 5 substitutions per 100 nucleotide positions.

multistilata branches separately from its supposed congener *Anteholosticha manca*, which is invariably placed in the Urostylida core clade.

T. brachysticha branches as sister group to the genus *Parabirojimia* clade with very high bootstrap values (ML, 100%; BI, 1.0; NJ, 100) within the newly established family

Parabirojimiidae (Fig. 4). *Trachelostyla pediculiformis* consistently clusters with Parabirojimiidae (ML, 55%; BI, 0.64; NJ, 72%). Both *Trachelostyla* and Parabirojimiidae branch as sister to Urostylida and other core Stichotrichia in the ML and BI trees (ML, 77%; BI, 0.97) while, in the NJ tree, they branch at the base of the Sporadotrichida group, but with low support (52%).

DISCUSSION

T. brachysticha and *T. multinucleata* as novel species

The two ciliates isolated from marine sediments obviously belong to the genus *Tunicothrix*, which is characterized by the conspicuous AL, the distinctly bipartite AZM and the urostylid midventral pairs confined to the oral region. *T. brachysticha* is very similar to *T. wilberti* (Fig. 1h, i). However, *T. brachysticha* is distinguished by the very short midventral row of only 8–13 (vs 20–36) cirri terminating in the anterior body half (anterior 40% vs posterior 60%) and the right marginal row 1 of 16–22 (vs 31–47) cirri restricted at mid-body portion (vs near body end). It is the two distinctly shortened rows that provide *T. brachysticha* with a different configuration from *T. wilberti* as well as *T. rostrata* (Fig. 1j, k), that is, a large blank area in the ventral field (Figs 1a, c, d and 2d, e, h). In addition, *T. brachysticha* has a beak-like frontal protrusion that is not mentioned for *T. wilberti* (Lin & Song, 2004). However, one should not overinterpret such an inconspicuous structure that might have been overlooked in *T. wilberti*. In comparison to *T. rostrata*, *T. brachysticha* is smaller (*in vivo* about 90×30 vs 160×40 μm) and possesses markedly fewer adoral membranelles and ventral cirri (Table 1). Moreover, *T. rostrata* has a unique elongated right marginal row 2 curving onto the anterior dorsal end of the cells (vs shortened on ventral side in congeners). *T. multinucleata* differs distinctly from all congeners in possessing more (7–10, usually eight) than two macronuclear nodules. Like *T. brachysticha*, the species has a much more shortened midventral row and right marginal row 1 (Figs 1e–g and 3e, f).

The discovery of the two novel species greatly increases our knowledge of the biodiversity of this ciliate genus. It is noteworthy that all four species of *Tunicothrix* have so far been found only in coastal regions of the Yellow Sea. Both *T. brachysticha* and *T. multinucleata* were discovered in intertidal sediments, while *T. rostrata* and *T. wilberti* were found in coastal waters. *T. multinucleata* has a rather slender body shape, well adapted to living in soft sediments. Likewise, the distinctly shortened midventral and right marginal rows in the two novel ciliates are also likely to represent adaptations to life in intertidal sediments. Of special interest is the presence of many striking, arc-shaped structures, 10–20 μm long, in all known species of *Tunicothrix*. Unfortunately, we have not been able to decide whether these structures are intrinsic or belong to an endosymbiont of the cell.

Parabirojimidae n. fam.

Diagnosis. Marine Urostylida with a roughly T-shaped bipartite AZM, three enlarged frontal cirri and a midventral complex with midventral pairs confined to buccal field.

Type genus. *Parabirojimia* Hu, Song & Warren, 2002.

Nomenclature. Yi *et al.* (2008) already suggested a family Parabirojimidae to include the genus *Parabirojimia*, but without any characterization. Thus, it is a nomen nudum according to Article 13.1 of the ICZN (1999).

Based on morphological and molecular data, the family Parabirojimidae contains the two genera *Tunicothrix* and *Parabirojimia*. *Tunicothrix* differs distinctly from *Parabirojimia* in having a conspicuous cortical AL (vs absent) and invariably two (vs no less than five) right marginal rows (Hu *et al.*, 2002; Xu *et al.*, 2006). Moreover, *Tunicothrix* has a minute but peculiar feature: the anteriormost cirrus of the left marginal row is separated from the other cirri by different polarization (Figs 1c, d, f, g and 2g). Xu *et al.* (2006) mentioned this feature but did not rank it as a generic character. The distinct anteriormost cirrus probably also exists in *Parabirojimia* (Chen *et al.*, 2010; Hu *et al.*, 2002; Lin & Song, 2004). Whether this feature is a synapomorphy for the family Parabirojimidae needs further careful investigation.

Tunicothrix and *Parabirojimia* were originally assigned to the family Urostylidae based on morphological characters (Hu *et al.*, 2002; Xu *et al.*, 2006). Berger (2006) assigned *Parabirojimia* with *Australothrix*, *Bakuella*, *Birojimia*, *Holostichides*, *Metaurostylopsis* and *Paragastrostyla* to the urostylid family Bakuellidae. Parabirojimidae differs distinctly from Bakuellidae by the possession of the unique roughly T-shaped bipartite AZM. The establishment of Parabirojimidae is also well supported by molecular phylogenetic trees, where *Tunicothrix* always clusters with *Parabirojimia*, forming a clade separate from all other urostylids.

Without the inclusion of *Tunicothrix*, Chen *et al.* (2010) constructed a phylogenetic tree in which *Parabirojimia* branches as a sister group to the Oligotrichia + Choreotrichia clade instead of the Stichotrichia clade. This topology might have been an artefact of under-sampling, since some crucial taxa, in particular *Tunicothrix* and *Trachelostyla*, were lacking. Nonetheless, the phylogenetic relationship between the new family and other urostylids is not well resolved. As indicated already by Foissner *et al.* (2004) and Schmidt *et al.* (2007), the phylogenetic relationships of Stichotrichia are rather complicated (e.g. the confusing Sporadotrichida and Urostylida assemblages). To date, almost all molecular trees including the Stichotrichia are rather vague and therefore should not be overinterpreted. For instance, *Anteholosticha multistilata* clusters with the Urostylida core clade in our BI tree, as well as in those of Yi *et al.* (2008), Chen *et al.* (2010) and Liu *et al.* (2010), while it branches within the Sporadotrichida in our ML and NJ trees. Moreover, *Anteholosticha multistilata* never clusters with its supposed congener *Anteholosticha manca* in any molecular tree. Likewise, *Trachelostyla pediculiformis* is always separated from the supposedly related *Gonostomum*, but invariably clusters with the *Tunicothrix* + *Parabirojimia* clade in all phylogenetic trees, a topology that obviously

contradicts those inferred from morphology and morphogenesis (Gong *et al.*, 2006; Shao *et al.*, 2007). Whether the situation is due to the lack of sequences of *Trachelostyla* or the lack of crucial taxa in Stichotrichia requires further investigation.

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