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Redescription of *Triplumaria selenica* Latteur et al., 1970 (Ciliophora, Entodiniomorphida) and its phylogenetic position based on the infraciliary bands and 18SSU rRNA gene sequence

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Abstract

Triplumaria selenica Latteur, Tuffrau and Wespes, 1970 was redescribed from pyridinated silver carbonate-impregnated specimens. *Triplumaria selenica* has a slit of the vestibular opening extending posteriorly along the left side of the vestibulum. The wide C-shaped adoral polybrachykinety extends along the ventral side of the vestibular opening. The narrow perivestibular polybrachykinety extends laterally along the dorsal side of the vestibular opening from the right end of the adoral polybrachykinety and forms a loop extending posteriorly along the vestibular slit to join to the left end of the adoral polybrachykinety. The 18SSU rRNA gene of *T. selenica* as well as those of six other entodiniomorphid species, *Raabena bella*, *Blepharocorys curvigula*, *Entodinium longinucleatum*, *Eudiplodinium rostratum*, *Metadinium medium*, and *Ostracodinium gracile* was sequenced. The neighbor joining and maximum parsimony phylogenetic trees were constructed to discuss the evolution of entodiniomorphs. Our results will support and extend Wolska's hypothesis: the ancestral forms of blepharocorythids have evolved into ophryoscolecids and *Cycloposthium* species via the ancestor of *Triplumaria*.

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Keywords: 18SSU rRNA; Blepharocorythidae; Entodiniomorphida; infraciliature; *Triplumaria selenica*; Ophryoscolecidae

Introduction

Ciliates in the genus *Triplumaria* Hoare, 1937, order Entodiniomorphida, Class Litostomatea, are found in the intestine of elephants and rhinoceroses (Eloff and

Van Hoven 1980; Hoare 1937; Latteur et al. 1970; Timoshenko and Imai 1995; Van Hoven et al. 1998). *Triplumaria selenica* was first described from African elephants and has been later also found in Asian elephants and African white rhinoceroses. *Triplumaria selenica* has relatively wide distribution in elephants (Timoshenko and Imai 1995) and the original description of *T. selenica* was incorrect and the 18SSU rRNA

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gene sequence of this species has been unknown. To discuss phylogenetic relationship between *T. selenica* and other entodiniomorphs, we will redescribe *T. selenica* using the pyridinated silver carbonate impregnation and will sequence the 18SSU rRNA of *T. selenica*. Phylogenetic trees will be constructed from 18SSU rRNA gene sequences of *T. selenica*, *Raabena bella*, *Blepharocorys curvigula*, *Entodinium longinucleatum*, *Eudiplodinium rostratum*, *Metadinium medium*, *Ostracodinium gracile*, other 11 entodiniomorphs, five vestibuliferids, and 10 macropodiniids.

Materials and methods

Samples

A fecal sample for *Triplumaria selenica* Latteur, Tuffrau and Wespes, 1970 and *Raabena bella* Wolska, 1967 was obtained from an Asian elephant kept in Oji zoo in Hyogo pref, Japan and a fecal sample for *Blepharocorys curvigula* Gassovsky, 1919 from a riding horse kept in Izmir, Turkey. A sample of rumen contents for *Entodinium longinucleatum* Dogiel, 1925 was obtained from a Japanese black beef cattle after slaughtered in Shimane pref and a sample of rumen contents for *Eudiplodinium rostratum* (Fiorentini, 1889), *Metadinium medium* Awerinzew and Mutafova, 1914, and *Ostracodinium gracile* (Dogiel, 1925) was obtained from a Holstein Friesian cattle by a rumen catheter in Miyazaki pref, Japan. *Entodinium longinucleatum* has four morphotypes depending on its caudal spines (Dehority 1979; Dogiel 1927; Imai 1984; Kofoid and MacLennan 1930; Williams and Coleman 1992) and cells examined herein were non-spined *Ent. longinucleatum*.

For the purpose of the morphological study using the silver impregnation, the fecal sample of the elephant was immediately fixed in five times the volume of 10% formalin solution within 5 min after defecation and was stored in a dark place after it was filtered through two layers of gauzes into a bottle to remove plant and feed material. Samples for the 18SSU rRNA gene sequences were fixed in five times the volume of 80% ethanol within 5 min after sampling from host animals and preserved in the freezer (4°C) after they were filtered through two layers of gauzes and their supernatant was replaced with 100% ethanol.

Morphology and silver impregnation

The infraciliary bands of *T. selenica* were stained by the pyridinated silver carbonate impregnation method, following Ito and Imai (1998). The orientation of ciliates used by Dogiel (1927) was adopted; the side beneath

which the macronucleus lies was termed the dorsal side; the opposite one the ventral side; defining the right and left sides. Cell measurements were made from a sample of 20 fixed cells using a calibrated micrometer. Body length was determined as the distance between the anterior and posterior ends of the body. The term, polybrachykinety, refers to infraciliary bands composed of numerous, short, parallel kineties (Ito and Imai 1998; Fernández-Galiano et al. 1985). Permanent slides of *T. selenica* are deposited in The National Science Museum, Tokyo, Japan (accession number NSMT-Pr 256).

DNA extraction and amplification

DNA extraction of *T. selenica*, *R. bella* and *B. curvigula* was performed according to the CTAB protocol for rumen ciliates by Wright et al. (1997). Each 60 cells of *T. selenica*, *R. bella* and *B. curvigula* were collected under an inverted microscope using a micromanipulator and a microinjector. PCR amplifications of *T. selenica* were performed in 50 µl volume, containing of template DNA in 5 µl, 0.25 µM of both forward and reverse primers (5'-AAC CTG GTT GAT CCT GCC AGT-3') and (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3'; Medlin et al. 1988), 0.2 mM each dNTP Mixtures, 2 mM MgCl₂, and 0.375 U of Takara Ex Taq DNA polymerase (Takara Bio, Otsu, Japan) with the following PCR program: 1 cycle at 95 °C for 5 min; 20 cycles at 98 °C for 5 s, 50 °C for 1 min, 72 °C for 2 min; and 1 cycle at 72 °C for 5 min. PCR amplifications of *R. bella* and *B. curvigula* were performed in 50 µl volume, containing of template DNA in 5 µl (*R. bella*) and in 3 µl (*B. curvigula*), 0.40 µM of both the same forward and reverse primers as described above, 0.2 mM each dNTP Mixtures, 1 mM MgCl₂, and 1.25 U of Takara PrimeSTAR GXL DNA polymerase (Takara Bio, Otsu, Japan) with the following PCR program: 1 cycle at 98 °C for 1 min; 30 cycles at 98 °C for 10 s, 55 °C for 15 s, 68 °C for 2 min. The PCR products of *T. selenica*, *R. bella* and *B. curvigula* were evaluated by electrophoresis in a 1% agarose gel followed by staining with ethidium bromide solution and visualization on ultraviolet transilluminator. The PCR products purified by electrophoresis were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 3730xl Genetic Analyzer (Applied Biosystems).

DNA extraction of *Ent. longinucleatum*, *Eud. rostratum*, *M. medium* and *O. gracile* was performed using the freeze-thawing single-cell PCR method of Honma et al. (2007). Single cell was washed three times in distilled water and drawn up in a volume of 1 µl distilled water and transferred to a PCR tube with a micropipette under the inverted microscope. The PCR tube was frozen at -80 °C for 3 min and thawed at 60 °C for 30 s

to facilitate cell breakage. This freeze-thawing step was repeated five times. PCR amplifications were performed in 15 μ l volume, containing of template DNA in 1 μ l distilled water, 0.25 μ M of both the same forward and reverse primers as described above, 0.2 mM each dNTP Mixtures, 2 mM MgCl₂, and 0.375 U of Takara Ex Taq DNA polymerase with the following PCR program: 1 cycle at 94 °C for 5 min; 40 cycles at 98 °C for 10 s, 50 °C for 30 s, 72 °C for 2 min; and 1 cycle at 72 °C for 10 min. The PCR products were evaluated by electrophoresis in a 1.2% agarose gel followed by staining with ethidium bromide solution and visualization on ultraviolet transilluminator. The PCR products were purified using MagExtractor-PCR & Gel Clean up (Toyobo, Osaka, Japan). The purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit on an ABI 3130xl Genetic Analyzer.

Sequence availability

The 18SSU rRNA gene sequences in the present study are available from the GenBank database. The accession numbers are as follows: *Amylovorax dehorityi* AF298817 (Cameron et al., 2001), *Amylovorax dogieli* AF298825 (Cameron et al., 2001), *Balantidium coli* AF029763 (Strüder-Kypke et al., 2006), *Bandia cribbi* AF298824 (Cameron and O'Donoghue, 2004), *Bandia smalesae* AF298822 (Cameron and O'Donoghue, 2004), *Bandia tamar AF298823* (Cameron and O'Donoghue, 2004), *Bitricha tasmaniensis* AF298821 (Cameron and O'Donoghue, 2004), *Cochliotaxum periachtum* EF632078 (Strüder-Kypke et al., 2007), *Cycloposthium edentatum* EF632077 (Strüder-Kypke et al., 2007), *Cycloposthium ishikawai* EF632076 (Strüder-Kypke et al., 2007), *Dasytricha ruminantium* U27814 (Embley et al., 1995), *Didinium nasutum* U57771 (Wright and Lynn, 1997a), *Diplodinium dentatum* U57764 (Wright and Lynn, 1997b), *Entodinium caudatum* U57765 (Wright et al., 1997), *Epidinium caudatum* U57763 (Wright et al., 1997), *Eudiplodinium maggii* U57766 (Wright and Lynn, 1997b), *Isotricha intestinalis* U57770 (Wright and Lynn, 1997a), *Isotricha prostoma* AF029762 (Wright and Lynn, 1997b), *Loxophyllum utriculariae* L26448 (Leipe et al., 1994), *Macropodinium ennuensis* AF298820 (Cameron et al., 2003), *Macropodinium yalanbense* AF042486 (Cameron et al., 2003), *Ophryoscolex purkynjei* U57768 (Wright and Lynn, 1997b), *Paraisotricha colpoidea* EF632075 (Strüder-Kypke et al., 2007), *Polycosta roundi* AF298819 (Cameron and O'Donoghue, 2004), *Polycosta turniae* AF298817 (Cameron and O'Donoghue, 2004), *Polyplastron multivesiculatum* U57767 (Wright et al., 1997), *Tripalmaria dogieli* EF632074 (Strüder-Kypke et al., 2007), *Troglodytella abrassarti* AB437346 (Irbis et al., 2008).

Phylogenetic analysis

For phylogenetic analysis, 18SSU rRNA gene sequences from *T. selenica*, *R. bella*, *B. curvigula*, *Ent. longinucleatum*, *Eud. rostratum*, *M. medium*, *O. gracile*, 11 entodiniomorphs, five vestibuliferids, and 10 macropodiids were aligned using ClustalW 1.83 (Thompson et al. 1994). Neighbor joining (Saitou and Nei 1987) and maximum parsimony (Ferris 1970) analyses were conducted in MEGA4 (Tamura et al. 2007). The evolutionary distances in the neighbor joining (NJ) tree were calculated using the maximum composite likelihood method (Tamura et al. 2004). The maximum parsimony (MP) tree was computed using the close-neighbor-interchange algorithm (Nei and Kumar 2000) with search level 3 (Felsenstein 1985). Both distance and parsimony data were bootstrap resampled 1000 times (Felsenstein 1985).

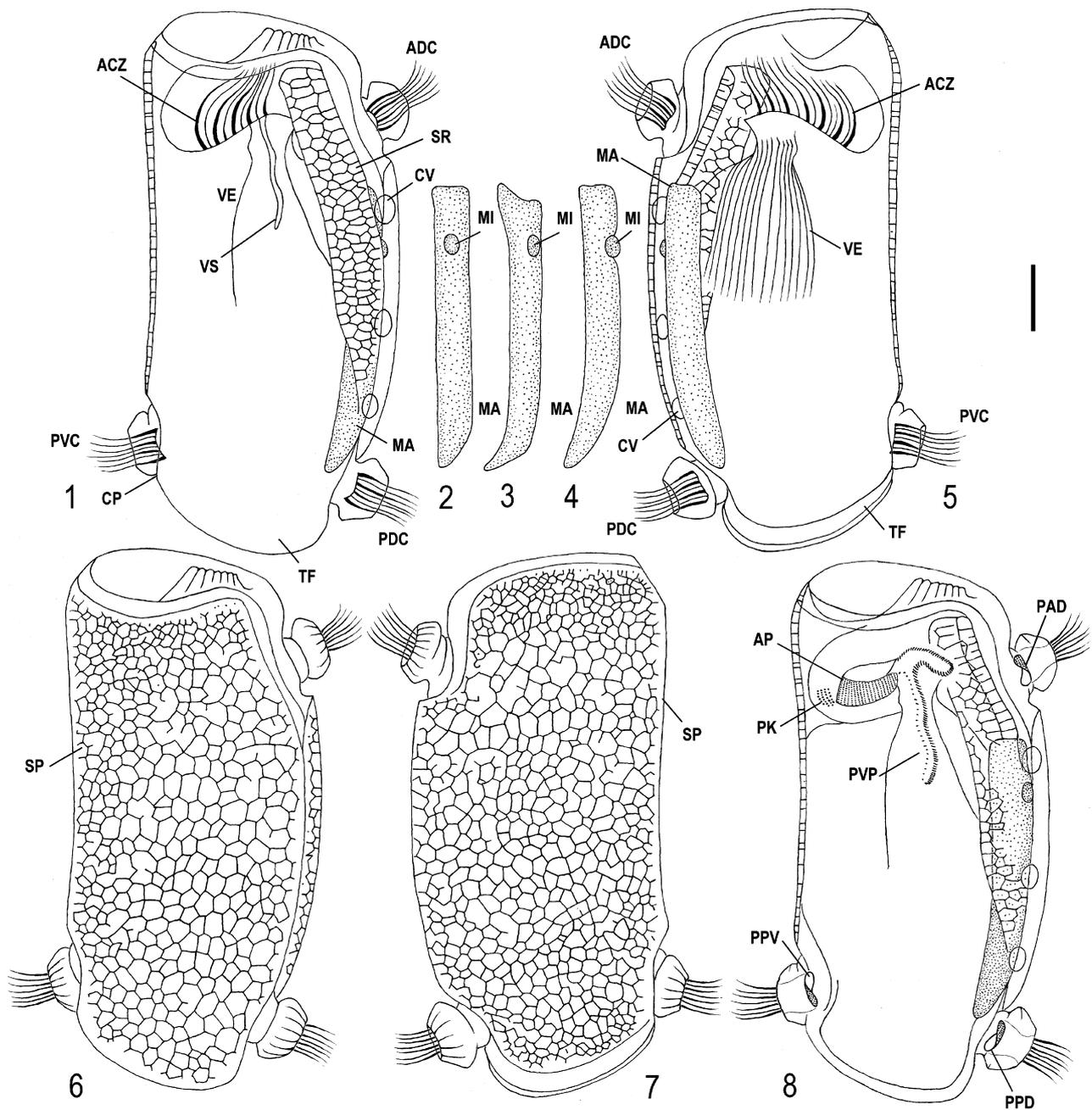
Results

General morphology of *Triplumaria selenica*

(Table 1, Figures 1–7). The body is rectangular with a round tail flap and two dorsal and one ventral mushroom-shaped caudalia, laterally compressed. The dorsal surface of the body is convex and the ventral surface is straight. The surface of the body protrudes anteriorly, forming an anterior collar which encircles the

Table 1. Measurements (μ m) and morphometric ratios (upper line: mean \pm SD; lower line: minimum–maximum; n = 20) of *Triplumaria selenica*.

Body length	167.0 109.7	\pm -	33.7 209.1
Body width	76.8 48.5	\pm -	17.0 96.9
Body length/body width	2.19 1.95	\pm -	0.18 2.57
Macronuclear length	88.9 63.8	\pm -	14.5 114.8
Macronuclear length/ body length	0.54 0.46	\pm -	0.05 0.62
Distance from anterior end of the macronucleus to the micronucleus	18.5 10.2	\pm -	6.6 30.6
Distance from anterior end of the macronucleus to the micronucleus/ macronuclear length	0.20 0.13	\pm -	0.05 0.30



Figures 1–8. Schematic figures of *Triplumaria selenica*. 1. Cell from left side. 2–4. Macronucleus and micronucleus from left side. 5. Cell from right side. 6. Skeletal plate seen from left side. 7. Skeletal plate seen from right side. 8. Cell from left side after silver impregnation. ACZ, adoral ciliary zone; ADC, anterior dorsal caudalium; AP, adoral polybrachykinety; CP, cytoproct; CV, contractile vacuole; MA, macronucleus; MI, micronucleus; PAD, polybrachykinety of anterior dorsal caudalium; PDC, posterior dorsal caudalium; PK, paralabial kineties; PPD, polybrachykinety of posterior dorsal caudalia; PPV, polybrachykinety of posterior ventral caudalium; PVC, posterior ventral caudalium; PVP, perivestibular polybrachykinety; SP, skeletal plate; SR, skeletal rod plate; TF, tail flap; VE, vestibulum; VS, vestibular slit. Bar = 20 μ m.

adoral lip. The right and left end of the anterior collar is round. A groove runs on the left dorsal surface of the body. A broad skeletal plate lying beneath the right, ventral, and left surfaces of the body is thick and consists of polygonal platelets. A skeletal rod plate extends from the anterior end of the body to near the

posterior dorsal caudalium beneath the left dorsal surface of the body. In the region anterior to the macronucleus of the skeletal rod plate, the dorsal side of the plate extends toward the right and, in posterior region, the plate extends along the left side of the macronucleus. The adoral ciliary zone is retractable into

the anterior end of the body. Non-retractable somatic ciliary arches arise from three caudalia. The anterior dorsal caudalium lies on the dorsal body surface at the level of the adoral ciliary zone and two posterior caudalia are situated at the dorsal and ventral bases of the tail flap. The vestibulum is hourglass-shaped with expanding vestibular opening and its posterior region. The vestibular opening extends posteriorly to form a slit along the left side of the vestibulum. The macronucleus is wedge-shaped, lying beneath the dorsal surface of the body, and varying in the shape. The micronucleus is small and ovoid, lying on the dorsal or right side of the anterior two-tenths of the macronucleus. The cytoproct is located behind the posterior ventral caudalium. Three contractile vacuoles lie beneath the dorsal surface of the body.

Infraciliature of *Triplumaria selenica*

(Figures 8, 9, 14). *Triplumaria selenica* has characteristic infraciliary bands in the buccal area. They are composed of an adoral polybrachykinety (AP), a perivestibular polybrachykinety (PVP), and paralabial kineties (PK). The C-shaped AP is wide and extends along the ventral side of the vestibular opening. The narrow PVP extends laterally from the right end of the AP along the dorsal edge of the vestibular opening and bends to extend posteriorly along the dorsal edge of the vestibular slit. Then the PVP turns back anteriorly along the ventral edge of the vestibular slit to join to the left end of the AP. In the PVP along the ventral edge of the vestibular slit,

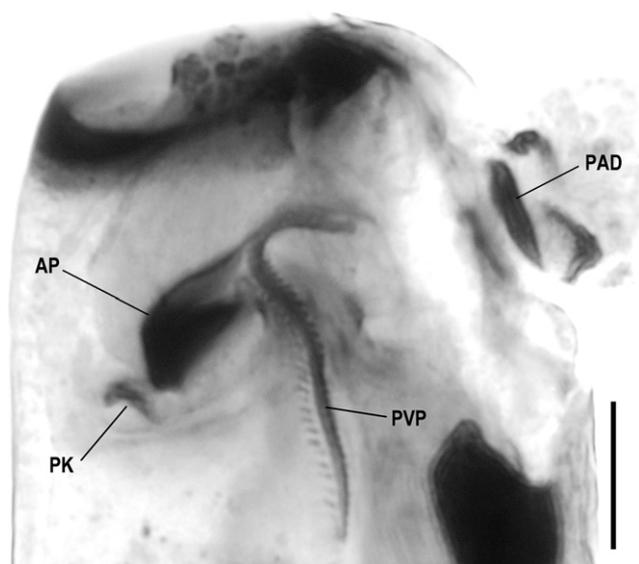


Fig. 9. Micrograph of *Triplumaria selenica* after pyridinated silver carbonate impregnation. Anterior half of the body from left side. AP, adoral polybrachykinety; PAD, polybrachykinety of anterior dorsal caudalium; PK, paralabial kineties; PVP, perivestibular polybrachykinety. Bar = 20 μ m.

each kinety row is arranged at wider spaced intervals. The PK is composed of more than four short transverse kineties extending along the ventral side of the AP. Kinetids in the PK are slightly larger than in kineties in other polybrachykineties.

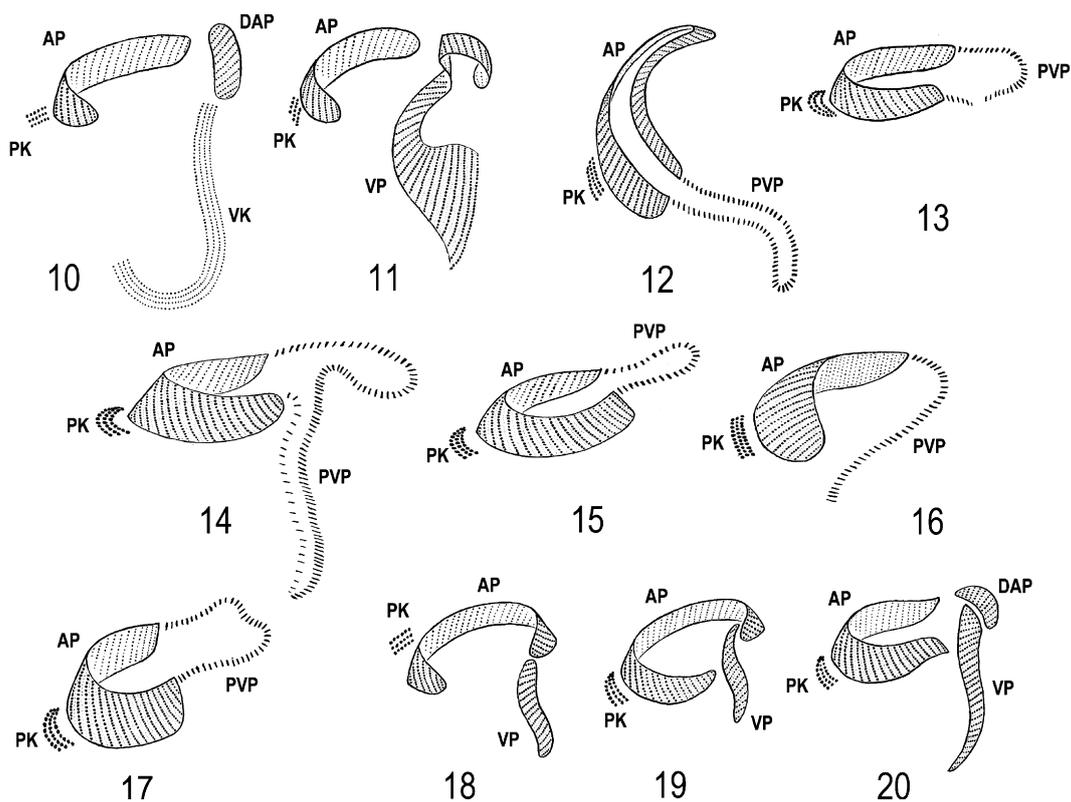
Triplumaria selenica has infraciliary bands of three caudalia on the dorsal and ventral surfaces; polybrachykinety of anterior dorsal caudalium (PAD), polybrachykinety of posterior dorsal caudalium (PPD), and polybrachykinety of posterior ventral caudalium (PPV). These three caudalial polybrachykineties are very short and nearly circular.

18SSU rRNA sequences

GenBank accession numbers and the length in base pairs are as follows: *Triplumaria selenica*, AB533538, 1639; *Raabena bella*, AB534183, 1640; *Blepharocorys curvigula*, AB534184, 1643; *Entodinium longinucleatum*, AB481099, 1594; *Metadinium medium*, AB535215, 1593; *Ostracodinium gracile*, AB535662, 1594; *Eudiplodinium rostratum*, AB536716, 1591.

Phylogenetic analysis

(Fig. 21). A total of 1470 unambiguously aligned sites were retained for phylogenetic analysis using ClustalW. To construct NJ and MP phylogenetic trees of 33 ciliates in the class Litostomatea using MEGA4, *Loxophyllum utriculariae* and *Didinium nasutum* were selected as outgroup. The NJ tree was drawn to scale in Fig. 21 with the bootstrap values at the nodes. The MP tree, one of eight most parsimonious trees, had the same branching order of the entodiniomorphid lineage as in the NJ tree and the bootstrap values of MP tree were shown in Fig. 21. Taxonomy of the order and the family in the class Litostomatea follows Lynn (2008). In the NJ and MP phylogenetic trees, the entodiniomorphs and the macropodiniids formed each a monophyletic group, whereas vestibuliferids were non-monophyletic. Trees could not be constructed with resolved branching order of the entodiniomorphid clades, after the 18SSU rRNA of seven entodiniomorphs were sequenced in the present study. *Entodinium longinucleatum*, *M. medium*, *O. gracile*, and *Eud. rostratum* and six other species in the family Ophryoscolecidae formed a clade, and ciliates in the genera *Cycloposthium* and *Troglodytella* clustered as sister group. These two clusters formed a terminal clade in the lineage of entodiniomorphs with relatively high bootstrap support for bifurcation, 58% in NJ tree and 65% in MP tree and formed a clade with *Triplumaria selenica* with high bootstrap support, 79% in NJ tree and 85% in MP tree. The family Cycloposthiidae is a non-monophyletic group. Two blepharocorythids, *Raabena bella* and *Blepharocorys curvigula*, branched



Figures 10–20. Schematic figures of buccal infraciliature of entodiniomorphs. **10, 11.** The Family Blepharocorythidae. **10.** *Blepharocorys curvigula*. From Wolska (1971a, b). **11.** *Raabena bella*. From Wolska (1967). **12.** *Cochliatoxum periachtum* in the family Spirodiniidae. Unpublished figure. See Wolska (1980). **13–17.** The family Cycloposthiidae. **13.** *Triplumaria dogieli*. From Wolska (1978). **14.** *Triplumaria selenica*. **15.** *Triplumaria alluvia*. From Ito et al. (2008). **16.** *Triplumaria grypoclunis*. From Ito et al. (2008). **17.** *Cycloposthium edentatum*. From Fernández-Galiano (1959). **18–20.** The family Ophryoscolecidae. **18.** *Entodinium longinuclatum*. Unpublished figure. See Ito et al. (2002). **19.** *Eudiplodinium rostratum*. From Ito et al. (2002). **20.** *Ostracodinium gracile*. From Ito and Imai (1998). AP, adoral polybrachykinety; DAP, dorso-adoral polybrachykinety; PK, paralabial kineties; PVP, perivestibular polybrachykinety; VK, vestibular kinety band; VP, vestibular polybrachykinety.

basally in the entodiniomorphid lineage with high bootstrap support, 81% in NJ tree and 77% in MP tree.

Discussion

Triplumaria is an entodiniomorphid genus which has skeletal plates beneath the body surface, a retractable adoral ciliary zone, and three additional somatic ciliary zones called “caudalium or caudalia” (Dehority 1986; Grain 1994; Kornilova 2004). *Triplumaria* is classified in the family Cycloposthiidae according to Lynn (2008), whereas it is one of four genera in the family Tripalmariidae in Grain’s system (1994). In the present study, neither *Cycloposthium* species nor *Triplumaria dogieli* formed a clade with *Triplumaria selenica*.

The description of *Triplumaria selenica* needs to be improved because of the incorrect original description as reported in our previous paper (Ito et al. 2008). Although the micronucleus was described to be located at the dorsal side of the macronucleus in Latteur et al.

(1970), it is frequently located at the left side of the macronucleus. The vestibular slit extending posteriorly is a remarkable feature which sets *T. selenica* apart from the other *Triplumaria* species and resembles the slit-like vestibular opening of *Cochliatoxum*, *Tetratoxum*, *Spirodinium* and others in the family Spirodiniidae. However, the vestibular slit in *T. selenica* was not described by Latteur et al. (1970).

Triplumaria resembles *Cycloposthium* species in the posterior part of the body and ophryoscolecids in the anterior part of the body (Hoare 1937; Latteur et al. 1970; Strelkow 1939; Timoshenko and Imai 1995). *Triplumaria selenica* with a thick skeletal plate resembles *Cycloposthium edentatum* and *C. bipalmatum*, whereas *T. ovinum* and *T. dvoinosi* with a very thin skeletal plate resemble *Diplodinium*, *Ostracodinium*, and others in the Ophryoscolecidae.

In addition to such external similarities, ciliates in the genus *Triplumaria* have close similarities in their infraciliary bands to those of ophryoscolecids, *Cycloposthium*, and other entodiniomorphs (Fernández-Galiano 1959; Ito et al. 2002, 2008; Wolska 1967, 1971b,

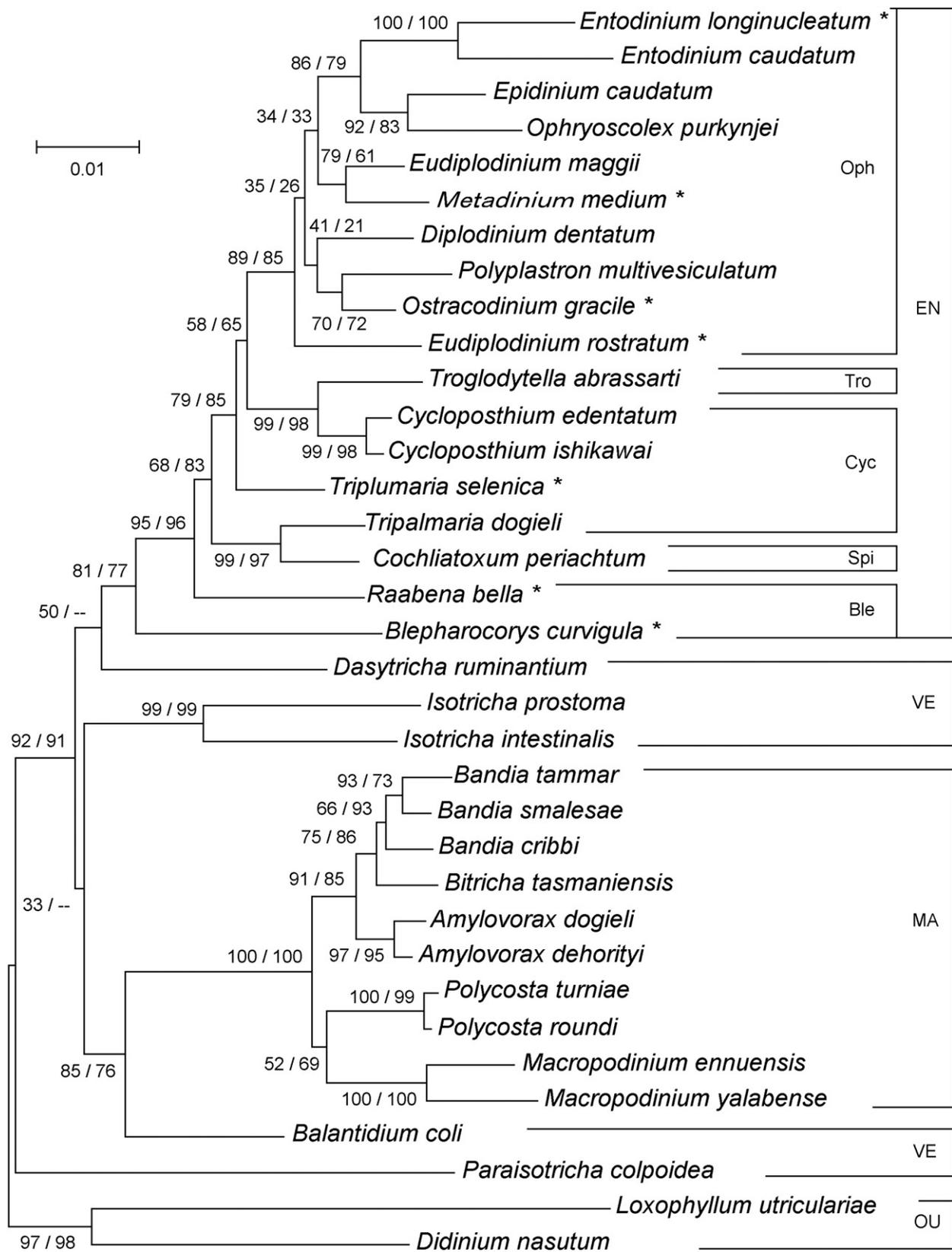


Fig. 21. Neighbor joining phylogenetic tree inferred from small subunit ribosomal RNA gene sequences. Species examined in the present study are asterisked. Bootstrap values (percent out of 1000 replicates) for neighbor joining and maximum parsimony are indicated at the nodes. The scale bar represents 1 change per 100 positions. Dashes represent nodes not existing in MP tree. Ble, Family Blepharocorythidae; Cyc, Family Cycloposthiidae; EN, Order Entodiniomorpha; MA, Order Macropodiniida; Oph, family Ophryoscolecidae; OU, Outgroup; Spi, Family Spirodiniidae; Tro, Family Troglodytelliidae; VE, Order Vestibuliferida.

1978, 1980). The PVP extending posteriorly in *T. selenica* (Fig. 14) is similar to the VP of ciliates in the Ophryoscolecidae (Figures 18–20). The PVP along the vestibular slit in *T. selenica* (Fig. 14) is analogous to the PVP of ciliates in the family Spirodiniidae (Fig. 12). By contrast, the PVP extending laterally along the dorsal side of the vestibular opening in *T. selenica* (Fig. 14) is homologous to the PVP of *Cycloposthium* species (Fig. 17). The AP and the PVP of *T. alluvia* (Fig. 15, Ito et al. 2008) are the same as found in *Cycloposthium* species (Fig. 17): their PVP extends along the dorsal edge of the vestibular opening to join to both right and left ends of the AP. The PVP of *T. grypochunisi* (Fig. 16, Ito et al. 2008), which is connected to the right end of the AP, is similar to the VP of ciliates in the Ophryoscolecidae (Figures 18–20).

Before the 18SSU rRNA gene of *Blepharocorys curvigula* (Fig. 10) and *Raabena bella* (Fig. 11) were sequenced in the present study, ciliates in the Blepharocorythidae had not been examined on their 18SSU rRNA. Wolska (1971a) reflected about the evolution of entodiniomorphs based on the infraciliary structures using silver impregnation and discussed that the early branching forms of the family Buetschliidae or of the family Blepharocorythidae would be ancestral forms of ophryoscolecids, not of *Cycloposthium*. The 18SSU rRNA of ciliates in the family Buetschliidae remains unknown whereas ciliates in the family Blepharocorythidae can be regarded as early branching forms of the lineage of entodiniomorphs from the present results. In addition, both ophryoscolecids and *Cycloposthium* species formed a terminal clade in the lineage of entodiniomorphs and *T. selenica* branched basally from this clade, with high bootstrap supports.

In conclusion, our results support and extend Wolska's hypothesis. The ancestral forms of blepharocorythids would evolve into ophryoscolecids and *Cycloposthium* species via the ancestors of *Triplumaria*.

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