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ORIGINAL ARTICLE

Intestinal ciliates from the domestic horses (*Equus ferus caballus*) in South Africa, and microtubule cytoskeleton organization of the representatives of Spirodiniidae (Ciliophora, Litostomatea)

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Summary

The fauna of endobiotic ciliates from the gut of domestic horses in South Africa was investigated. Samples were collected from horses that were kept in stables, as well as free-grazing ones. Ciliates from 39 species belonging to 22 genera were found, most of them were common in the intestinal fauna of horses. In addition, *Spirodinium nanum*, suggested to be specific for zebras, and *Blepharosphaera ceratotherii*, previously found in rhinoceros and zebras, were found. Immunofluorescent staining was used to study the organization of the microtubule cytoskeleton in some endobiotic ciliates. The structure of the ciliature of *Ditoxum funinucleum* is described for the first time. Conclusions are made about peculiarities of the organization of microtubule cytoskeleton in spirodiniids in the context of the taxonomy of this group of trichostomatids.

Key words: endobiotic ciliates, Trichostomatia, *Equus ferus caballus*, microtubule cytoskeleton, Spirodiniidae

Introduction

The species diversity of ciliates inhabiting the intestinal tract of the domestic horse *Equus ferus caballus* has been studied fairly well, with a total of more than 70 species (trichostomatids and suctorians) being recorded from this host (Cedrola et al., 2019). Nevertheless, faunistic studies of equine endobiotic ciliates forming local populations in different geographic regions remain a promising direction of research. They make it possible to assess how the structure of endobiotic communities is influenced by the size of the host population and the degree of its isolation as well as by various environmental factors. In some cases, a comparison of faunistic lists of ciliates from the intestines of horses from different geographic regions sheds light on the ways of formation of local horse populations.

The fauna of equine intestinal ciliates is rather specific, though some species have been found in other odd-toed ungulates (Kornilova, 2006; Vdachny, 2018). At the same time, it is still unknown whether different *Equus* spp. harbour species-specific endobiotic ciliates. In this respect,

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it would be particularly important to examine the composition of intestinal ciliate communities in various *Equus* spp. co-occurring in natural habitats, where transmission of endobionts across different species is possible.

We investigated the fauna of intestinal ciliates of domestic horses *E. ferus caballus* in South Africa. Some of the horses were kept in stables, while others were free grazing. In the latter case, their grazing grounds were located close to a nature reserve where the mountain zebras *Equus zebra* occur.

The ciliates from samples of horse faeces were identified with the use of various methods, including immunofluorescent staining of cytoskeleton elements formed by α-tubulin. Using this technique, the ciliature of *Ditoxum funinucleum* Gassovsky, 1919 was studied for the first time. We also performed a comparative analysis of the tubulin cytoskeleton organization in ciliates from the family Spirodiniidae (Lynn, 2008) in the context of taxonomic position of different genera therein.

Material and methods

Samples of faeces were collected from five horses (*E. ferus caballus*) at the southern slope of the Langeberg mountains near the town of Riversdale (Western Cape, South Africa, 33°58'60.0"S, 21°12' 45.6"E) in July 2019. An additional sample was collected from the horse living in the racing horse stable in Cape Town (Western Cape, South Africa, 33°59'50.8"S, 18°29'02.1"E). The samples were fixed by 96% ethanol within five minutes after defecation to prevent the destruction of intestinal ciliates.

The ciliates were stained by methyl green 1% solution in 1% acetic acid and by Lugol's iodine. We used an optical microscope MBI-11 and an optical inverted microscope Altami Invert-3 with an eyepiece micrometre for the preliminary examination of the samples. Ciliates were observed and photographed on glass object slides using a Leica DM 2500 microscope equipped with differential interference contrast (DIC).

Identification and taxonomy of ciliate species and genera was mainly based on the studies of Gassovsky (1919), Hsiung (1930), Strelkow (1931, 1939), Van Hoven et al. (1998), and Lynn (2008).

Microphotographs were taken with the Leica DFC495 (8.0MP) and Nikon Coolpix 4500 (4.0MP) digital cameras. The total number of ciliates in a fixed volume of liquid (100 µl) was counted on the slides. Since the number of ciliates in the samples was very low, we quantified the results in the following manner: + single specimen, ++ about 10 cells per ml, +++ many more than 10 cells per ml.

For immunofluorescent staining and microscopy, 50 µl of the sample were put on polylysine-coated slides and dried. After that, the slides were put into ice-cold methanol for 30 minutes and then washed in PBS thrice for 5 min each time, treated with 1% Triton X-100 for 20 min, washed in PBS thrice and blocked with 1% BSA for 10 min. Then 50 µl of primary antibodies (monoclonal anti-α-Tubulin antibodies produced in mouse (T5168, Sigma-Aldrich, USA) diluted with PBS 1:500) were added on the slides, which were then incubated at +4 °C overnight. Then the slides were washed thrice in PBS, and 50 µl of secondary antibodies Anti-Mouse IgG (whole molecule)–TRITC antibody produced in goat (Sigma-Aldrich T5393) (diluted with PBS 1:100) were added, incubated in the dark at room temperature for 1.5 h. The preparations were washed thrice in PBS and embedded into glycerine with addition of DAPI (1351303, Bio-Rad, USA) (2 µg/ml).

Large ciliate cells were picked individually using a glass pipette and placed in 2 ml microcentrifuge tubes in a small amount of liquid (50 µl). They were treated in the same way as the cells on the slide (see above). The reagents were changed using a micropipette. After staining, the cells were transferred onto glass and embedded in glycerol with the addition of DAPI. They were viewed under a Leica DM2500 microscope with a fluorescent module with the use of filter cube B/G/R, N2.1 and I3 (Leica-Microsystems, Wetzlar, Germany) and a Leica TCS SP5 laser confocal scanning microscope. The images were processed using ImageJ software.

Results and discussion

The total number of ciliates in the faecal samples of horses No 1, 5, and 6 did not exceed 5000 ciliates per ml, while that in horses No 2, 3, and 4 did not exceed 500 ciliates per ml. It should be noted that the number of ciliates in faecal samples may not correspond to the actual number of endobionts in the intestine since the latter is strongly influenced by various factors including the degree of hydration of the faeces. Noteworthy, the number of endobionts was also extremely low in faecal samples of the mountain zebra from similar habitats (Kornilova et al., 2020).

Table 1. List of species of intestinal ciliates of horses in South Africa.

No. gen.	No. spec.	Horse number Family/genus/subgenus/species	1	2	3	4	5	6
		Buetschliidae Poche, 1913						
1		*Alloiozona* Hsiung, 1930						
	1	*A. trizona* Hsiung, 1930	+		+	+		
2		*Blepharoconus* Gassovsky, 1919						
	2	*Blepharoconus sp.*	+	+				
3		*Blepharosphaera* Bundle, 1895						
	3	*B. ceratotherii* Van Hoven et al., 1998	+	+	+		+	+
4		*Holophryoides* Gassovsky, 1919						
	4	*H. ovalis* (Fiorentini, 1890)		+	+		+	+
	5	*H. macrotricha* Strelkow, 1939	+					+
5		*Polymorphella* Corliss, 1960						
	6	*P. ampulla* (Dogiel, 1929)	+	+			+	+
6		*Hemiprorodon* Strelkow, 1939						
	7	*H. gymnoposthium* Strelkow, 1939	+					+
7		*Blepharoprosthium* Bundle, 1895						
	8	*B. pireum* Bundle, 1895	+	+			+	+
8		*Bundleia* Cunha & Muniz, 1928						
		subgen. *Bundleia* Strelkow, 1939						
	9	*B. postciliata* (Bundle, 1895)	+++	+	+	+	+++	+++
	10	*B. piriformis* Strelkow, 1939	+					
	11	*B. nana* Strelkow, 1939		+	+			++
	12	*B. vorax* Strelkow, 1939					+	
		subgen. *Fibrillobundleia* Strelkow, 1939						
	13	*B. benbrooki* Hsiung, 1930	+++	+	+	+	+++	+++
	14	*B. inflata* Strelkow, 1939	+++	+	+	+		+++
	15	*B. dolichosoma* Strelkow, 1939		+		+		
9		*Prorodonopsis* Gassovsky, 1919						
	16	*P. coli* Gassovsky, 1919					+	
		Paraisotrichidae da Cuncha, 1917						
10		*Paraisotricha* Fiorentini, 1890						
	17	*P. minuta* Hsiung, 1930					+	
		Blepharocorythidae Hsiung, 1929						
11		*Blepharocorys* Bundle, 1895						
	18	*B. uncinata* (Fiorentini, 1890)					+	+
	19	*B. curvigula* Gassovsky, 1919	+++	+		+		+++
	20	*B. angusta* Gassovsky, 1919	++				+	+
	21	*B. microcorys* Gassovsky, 1919		+	+	+	+++	+++
	22	*B. valvata* (Fiorentini, 1890)	+	+	+		+	
12		*Ochoterenaia* Chavarria, 1933						
	23	*O. appendiculata* Chavarria, 1933	+				+	+
13		*Circodinium* Wolska, 1971						
	24	*C. minimum* (Gassovsky, 1919)	+		+		+	+
		Cycloposthiidae Poche, 1913						
14		*Cycloposthium* Bundle, 1895						
	25	*C. bipalmatum* (Fiorentini, 1890)					+	+

Table 1. Continuation.

	26	*C. edentatum* Strelkow, 1928	+++	+	+	+	+++	+++
	27	*C. dentiferum* Gassovsky, 1919						+
15		*Tripalmaria* Gassovsky, 1919						
	28	*T. dogieli* Gassovsky, 1919	+++			+		
		Spirodiniidae Strelkow, 1939						
16		*Ditoxum* Gassovsky, 1919						
	29	*D. funinucleum* Gassovsky, 1919	++			+	++	++
	30	*D. brevinucleatum* Strelkow, 1931	+					+
17		*Triadinium* Fiorentini, 1890						
	31	*T. caudatum* Fiorentini, 1890	+++	+		+	+++	+++
18		*Gassovskiella* Grain, 1994						
	32	*G. galea* (Gassovsky, 1919)	+++				+++	+++
19		*Cochliatoxum* Gassovsky, 1919						
	33	*C. periachtum* Gassovsky, 1919		+	+		+++	+++
20		*Tetratoxum* Gassovsky, 1919						
	34	*T. parvum* Hsiung, 1930	+	+	+		+++	+++
	35	*T. unifasciculatum* Fiorentini, 1890					+	
21		*Spirodinium* Fiorentini, 1890						
	36	*S. nanum* Strelkow, 1931		+	+		+	+
	37	*S. equi* Fiorentini, 1890	+				+	
	38	*S. confusum* Hsiung, 1935	+				+	+
		Allantosomatidae Jankowski, 1967						
22		*Allantosoma* Gassovsky, 1919						
	39	*A. intestinalis* Gassovsky, 1919	+	+	+	+		+
Total number of species			**25**	**19**	**15**	**12**	**26**	**28**

We found 39 species of intestinal horse ciliates belonging to 22 genera (Table 1; Figs 1–3). Most of the ciliate species were common endobionts of domestic horses. In general, species composition of ciliates in faecal samples of horse 1, which was kept in a stable, was similar to that in faecal samples of free-grazing horses 5 and 6. The samples of horses 2, 3, and 4 were disregarded because the numbers of ciliates in them were very low.

Blepharosphaera sp. found in our samples was identified as *B. ceratotherii* based on its size characteristics. Earlier, *B. ceratotherii* was found in the intestines of the rhinoceros and shown to be an independent species based on morphometry (van Hoven et al., 1998). Interestingly, the genus *Blepharosphaera* is also mostly represented by this species in zebra *E. zebra* and *E. quagga* from South Africa (Kornilova et al., 2020, 2021). It should be noted, however, that due to the great polymorphism of trichostomatids, the question of whether *B. ceratotherii* and *B. intestinalis*, a common equine endobiont, are indeed different species requires further investigation.

The presence of *Spirodinium nanum* in our samples is particularly interesting. This ciliate was described from the intestines of the zebra *Equus quagga* from Kenya (Strelkov, 1931). Nearly a century later, we identified these ciliates in the faeces of the mountain zebra *E. zebra* and the plains zebra *E. quagga* from South Africa (Kornilova et al., 2020, 2021). There is only one record of *S. nanum* from the domestic horse, and it was registered in Japan (Ike et al., 1985). We found this species in our study, in the faeces of free-grazing horses 2, 3, 5, and 6.

Transmission of equine endobiotic ciliates from one host to another occurs through coprophagy. Most trichostomatids do not form cysts and remain viable outside the host only for a very short time span. Therefore, transmission of endobionts, including transmission between different host species, is only possible when the hosts living in the same area come into close contact. We believe that the horses we studied may have acquired ciliates *S. nanum* as a result of contact with mountain zebras living in the reserve close to the grazing areas.

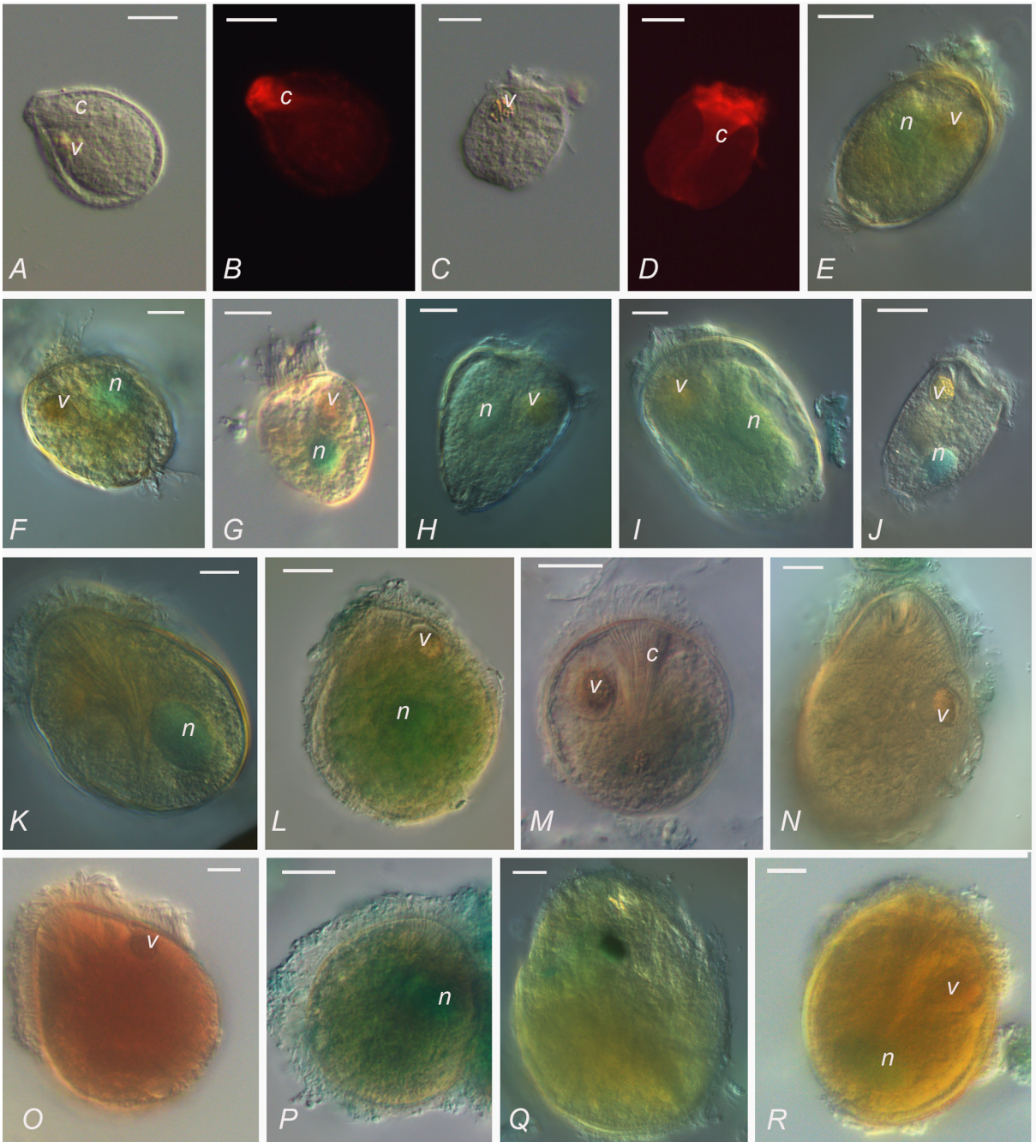

Fig. 1. Ciliates from the intestine of domestic horses in South Africa; family Buetschlidae. A, B – *Polymorphella ampulla*; C, D – *Bundleia benbrooki*; E – *B. inflata*; F – *B. postciliata*; G – *B. nana*; H – *B. piriformis*; I – *B. vorax*; J – *B. dolichosom;*, K – *Blepharoconus* sp.; L – *Hemiprorodon gymnoposthium*; M – *Blepharosphaera ceratotherii*; N – *Prorodonopsis coli*; O – *Blepharoprosthium pireum*; P – *Holophryoides macrotricha*; Q – *H. ovalis*; R – *Alloiozona trizona*. B, C – Immunofluorescent staining, other – DIC, E–L, P, R – staining by methyl green. *Abbreviations*: v – concrement vacuole, n – macronucleus, c – cytopharynx. Scale bars: 10 μm.

In this study, we investigated for the first time the ciliature of *Ditoxum funinucleum* Gassovsky, 1919 using immunofluorescent staining (Figs 4, 5). The oral ciliature of these ciliates consists of dorsal and ventral adoral ciliated zones (Fig. 4, B, C). The ventral zone forms an almost straight line, while the dorsal zone forms an arc. The general organization of the oral ciliature in *D. funinucleum* is similar to that

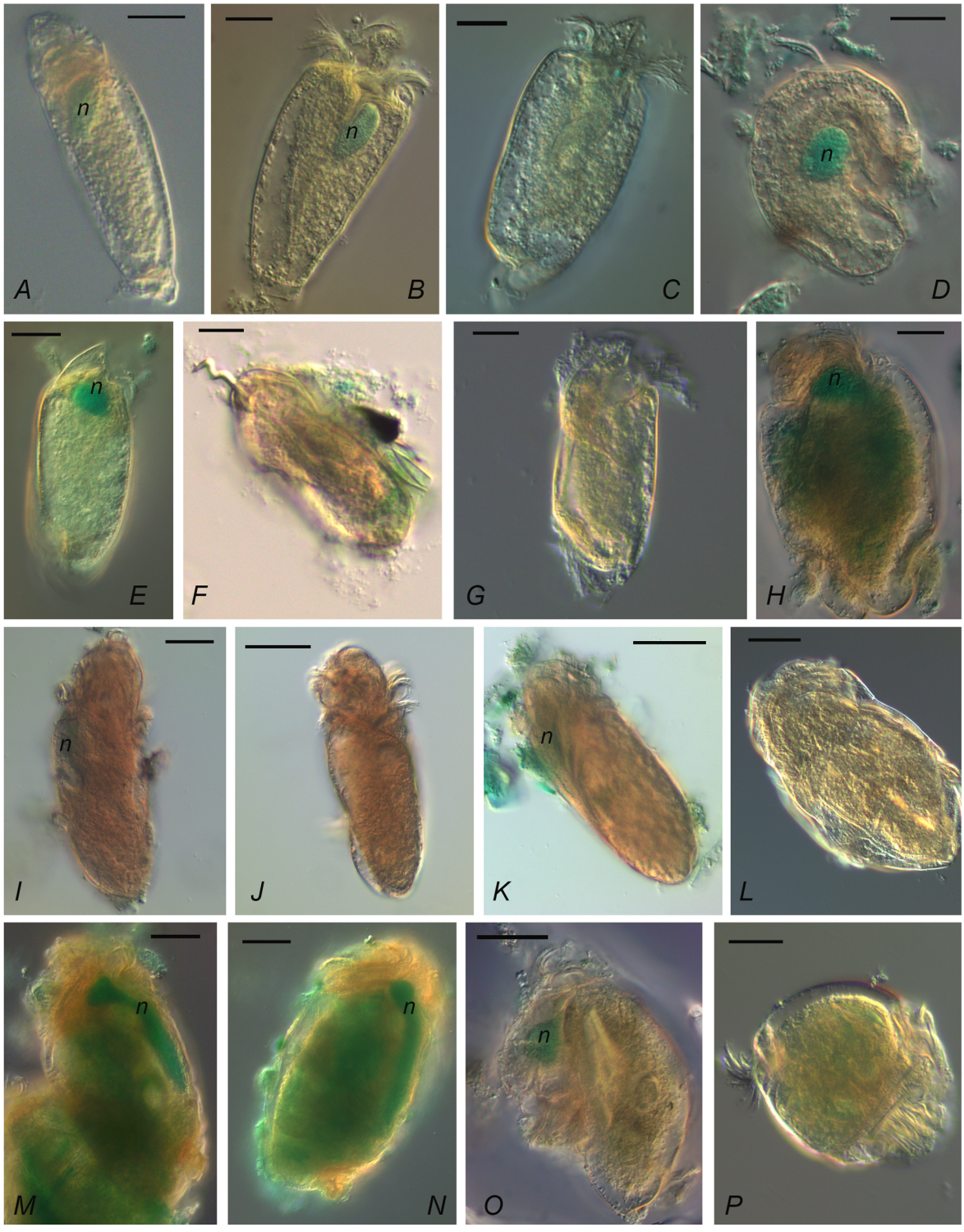

Fig. 2. Ciliates from the intestine of domestic horses in South Africa; families Blepharocorythidae and Spirodiniidae. A – *Blepharocorys angusta*; B – *B. curvigula*; C – *B. microcorys*; D – *Circodinium minimum*; E – *B. valvata*; F – *B. uncinata*; G – *Ochoterenaia appendiculata*; H – *Tetratoxum parvum*; I – *Spirodinium equi*; J – *S. confusum*; K – *S. nanum*; L – *Cochliatoxum periachtum*; M – *Ditoxum brevinucleatum*; N – *D. funinucleum*; O – *Triadinium caudatum*; P – *Gassovskiella galea*. A–P – DIC, A, B, D, E, H, I, K, M–O - staining by methyl green. *Abbreviations*: n – macronucleus. Scale bars: A - H - 10 μm, I - K, M – P - 20 μm, L – 50 μm.

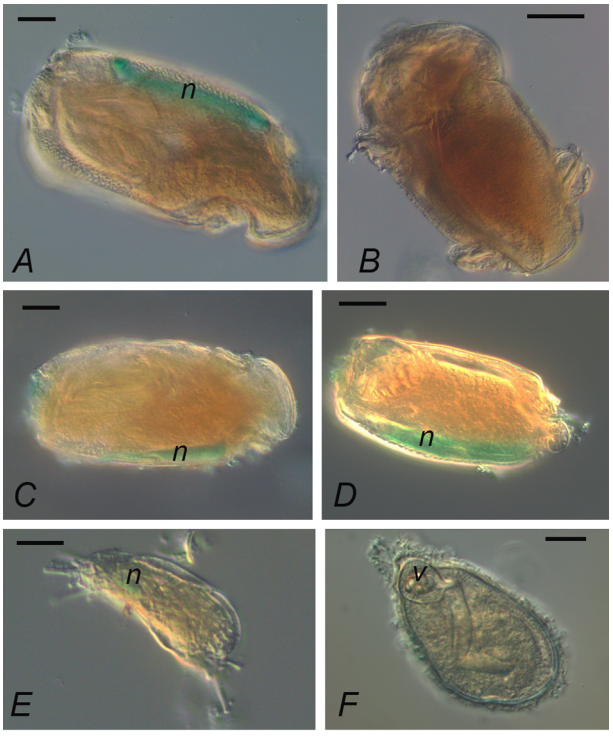

Fig. 3. Ciliates from the intestine of domestic horses in South Africa; families Cycloposthiidae, Allantosomatidae, and Paraisotrichidae. A – *Cycloposthium edentatum*; B – *Tripalmaria dogieli*; C – *C. dentiferum*; D – *C. bipalmatum*; E – *Allantosoma intestinale*; F – *Paraisotricha minuta*. A–F – DIC, A, C–E – staining by methyl green. *Abbreviations*: v – concrement vacuole, n – macronucleus. Scale bars: A–D – 20 μm, E, F – 10 μm

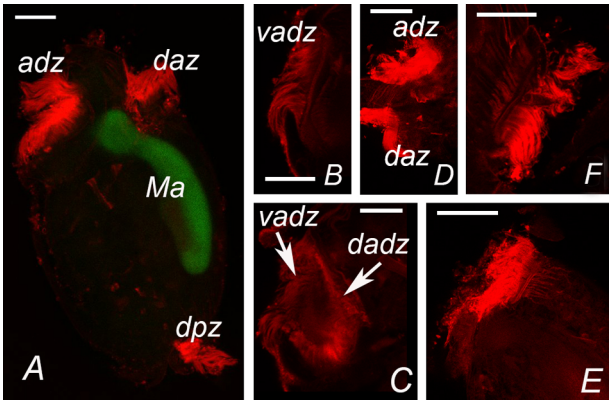

Fig. 4. Microtubule cytoskeleton organization in *Ditoxum funinucleum*, immunofluorescent staining. A – General morphology; B, C – adoral ciliature at different levels; D – bundles of microtubules between ADZ and DAZ; E – dorsal anterior ciliary zone; F – dorsal posterior ciliary zone. *Abbreviations*: Dadz – dorsal adoral zone, vadz – ventral adoral zone, adz – adoral zone, daz – dorsal anterior zone, dpz – dorsal posterior zone, Ma – macronucleus. CLSM, green colour corresponds to DAPI. Scale bars: A, D – E – 20 μm, B, C – 10 μm.

in *Tetratoxum* spp. and *Spirodinium* spp. (Wolska, 1980, 1985). In addition, anterior dorsal and posterior ventral somatic ciliated arches are clearly visible on the cell surface of the ciliates examined in our study (Fig. 4, A; Fig. 5). Regularly arranged long nemadesmata start from the somatic ciliated arches and pass towards the cell equator (Fig. 4, G, E). In some cases, parallel bundles of microtubules between the adoral zone and the anterior dorsal ciliary arch are also revealed (Fig. 4, D).

We also found a developed system of nemadesmata associated with somatic ciliated arches in *Cochliatoxum periachtum* and *Spirodinium equi* (Fig. 6, D, H–K). In these ciliates, the nemadesmata start from the ciliated arches and branch off in two directions. In *C. periachtum*, the two groups of nemadesmata associated with the anterior ciliated arch are equally well developed, while the two groups of the nemadesmata associated with the posterior ciliated arch are developed unequally, those directed towards the cell equator being particularly prominent (Fig. 6, I–K). In *S. equi*, in contrast to *C. periachtum*, regular nemadesmata extend from the posterior ciliated arch towards both the anterior and the posterior pole of the cell (Fig. 6, D, H, I). In addition to the system of nemadesmata, in some cases bundles of microtubules, arranged in parallel to each other along the longitudinal axis of the cell, are detected near the cell surface in this ciliate species (Fig. 6, E, F). Powerful bundles of nemadesmata extending in both directions from somatic ciliary arches were described also when studying the fine structure of *C. periachtum*, but TEM did not allow one to determine the general plan of arrangement of these cytoskeletal elements in the ciliate cell (Senaud and Grain, 1972).

In *Tetratoxum parvum*, regularly spaced nemadesmata associated with somatic ciliary arches were found only in cells that have begun to divide (Fig. 6, B). In other cases, solitary bundles of microtubules or a "brush" of short microtubules oriented perpendicular to the ciliated arch were seen (Fig. 6, A, C).

No bundles of microtubules associated with the somatic ciliature were found in *Triadinium caudatum* and *Gassovskiella galea* (Fig. 6, L–P). However, microtubular bundles connecting the adoral ciliary

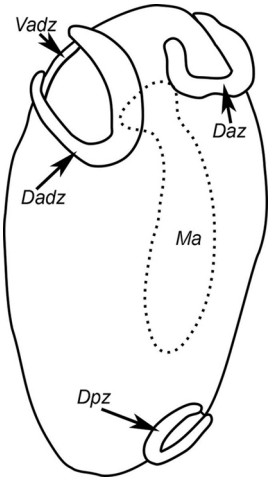

Fig. 5. Scheme of disposition of ciliary zones in *Ditoxum funinucleum*. *Abbreviations*: Dadz – dorsal adoral zone, vadz – ventral adoral zone, daz – dorsal anterior zone, dpz – dorsal posterior zone, Ma – macronucleus.

zone with the occipital zone and the so-called paralabial organ were sometimes detected in the cells of *T. caudatum* (Fig. 6, L, M).

It should be noted that the validity of including the genera *Cochliatoxum, Tetratoxum, Triadinium, Ditoxum*, and *Spirodinium* within the family Spirodiniidae has been debated for a long time. For instance, Wolska (1985) proposed to assign *Spirodinium* to a separate family on the basis of the structural features of the cortex and the arrangement of somatic ciliature. Jankowski (2007) proposed to transfer *Triadinium* and *Gassovskiella* to the order Blepharocorythidae based on the organization of oral ciliature (*Gassovskiella*, erected by Grain in 1994, is ignored in many classifications, including Lynn, 2008). Within the order Entodiniomorphida, Jankowski (2007) identified the family Ditoxidae (with *Ditoxum* and *Tetratoxum*) and the family Spirodiniidae (with *Spirodinium* and *Cochliatoxum*). According to the molecular phylogenetic analysis of 18S RNA sequences, all the genera form a single clade on the phylogenetic tree, although their mutual arrangement varies depending on the set of data (Ito et al., 2014; Vd'achny, 2018). At the same time, this clade also includes *Tripalmaria dogieli*, which, being considerably different morphologically, is traditionally included into the family Cycloposthiidae.

On the basis of our data we can conclude that *G. galea* and *T. caudatum*, on the one hand, and *D. funinucleum, C. periachtum*, and *S. equi*, on the other hand, differ not only in the organization of the oral ciliature but also in the structure of the tubulin cytoskeleton associated with the somatic cilia. *T. parvum* stands apart, being similar to *D. funinucleum, C. periachtum*, and *S. equi* in the structure of the oral ciliature but having no nemadesmata associated with the ciliated arches. We suggest that the organization of the microtubular derivatives associated with the somatic ciliature can be used as a differential character at the family level for this group of trichostomatid species.

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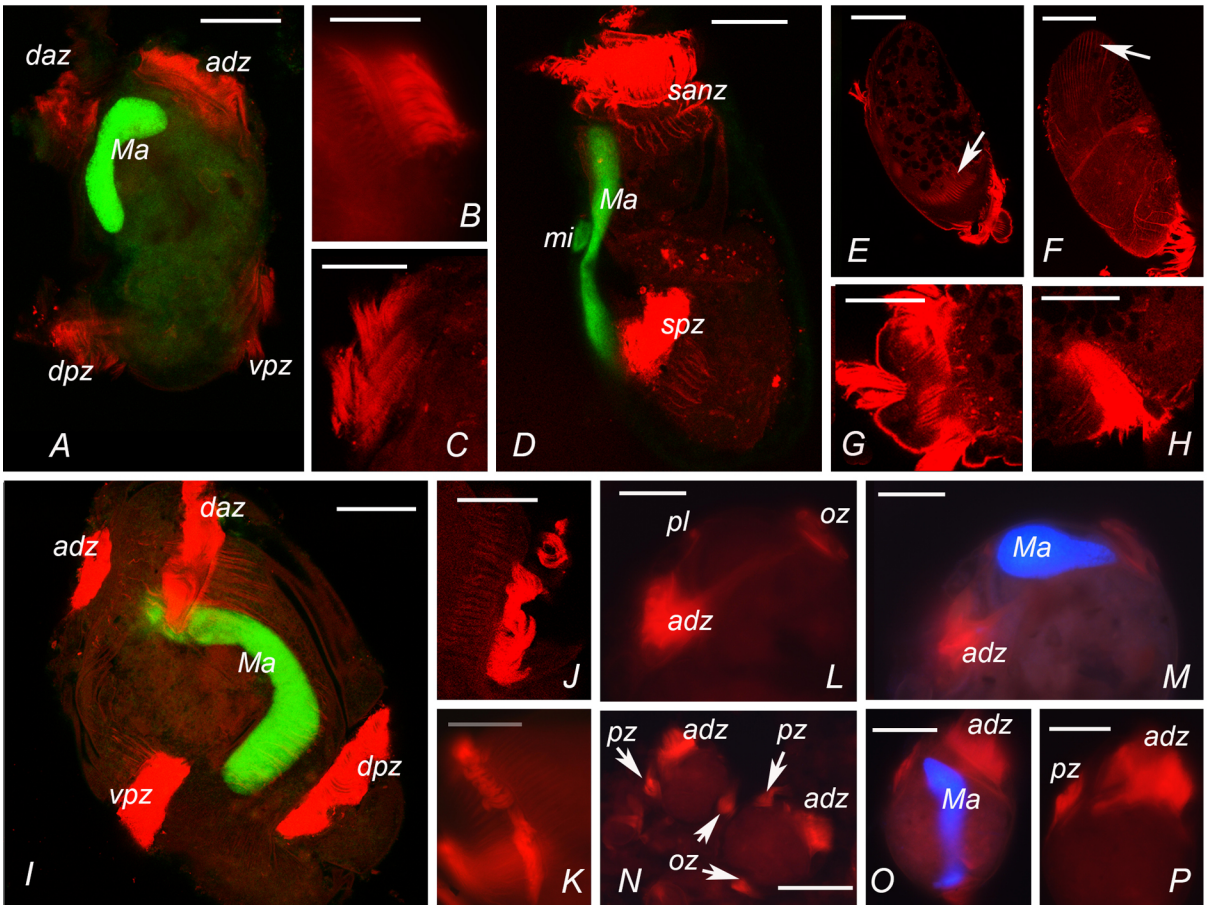

Fig. 6. Microtubule cytoskeleton organization in the representatives of Spirodiniidae, immunofluorescent staining. A–C – *Tetratoxum parvum* (A – general morphology, B – dorsal anterior ciliary zone, C – ventral posterior ciliary zone); D–G – *Spirodinium equi* (D – general morphology, E, F – bundles of microtubules near cell surface (*arrows*), G – anterior part of the cell); H – somatic posterior ciliary zone; I–K – *Cochliatoxum periachtum* (I – general morphology, J – dorsal posterior ciliary zone, K – dorsal anterior ciliary zone); L, M – *Triadinium caudatum*, N–P – *Gassovskiella galea*. *Abbreviations*: Adz – adoral zone, daz – dorsal anterior zone, dpz – dorsal posterior zone, vpz – ventral posterior zone, sanz – somatic anterior zone, spz – somatic posterior zone, pz – posterior zone, oz – occipital zone, pl – paralabial organelle, Ma – macronucleus, mi - micronucleus. A–K – CLSM, green colour corresponds to DAPI; L–P – fluorescent microscopy. Scale bars: A, B, D – H, J – M, O, P – 20 μm, C – 10 μm, I, N – 50 μm.

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