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ORIGINAL ARTICLE

Diversity of ciliates – endosymbionts of the intestine of Przewalski's horses from the Orenburg Nature Reserve

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Summary

Diversity of endobiotic ciliates from the intestine of Przewalski's horses from the Orenburg Nature Reserve was studied for the first time. In fecal samples of Przewalski's horses 20 species of ciliates belonging to 11 genera and 5 families were found. All identified ciliate species are typical for equids. No significant differences in ciliate species composition were found between Przewalski's horses of different origins and those living in free range and in corrals. It has been shown that free grazing horses have a considerably increased abundance of bacterivorous ciliates such as *Bundleia* sp., *Polymorphella ampulla*, and *Holophryoides macrotricha*. Przewalski's horses from the Orenburg Nature Reserve and domestic horses living in the same area differ significantly in the species composition of endobionts. The influence of the diet and social activity of the hosts on the structure of endobiotic ciliate communities, as well as the level of specificity of interaction between partners in the system are discussed.

Key words: endobiotic ciliates, diversity, communities, Przewalski's horses, specificity

Introduction

Endosymbiotic ciliates have been found in the digestive tract of many herbivorous mammals, primarily of even-toed and odd-toed ungulates (Cedrola et al., 2020). The assembling of endobionts in communities was studied mostly for rumen ciliates (Dogiel, 1927; Ogimoto and Imai, 1981; Williams and Coleman, 1992; Imai, 1998; Williams et al., 2020; Yu et al., 2025). In this case, the struc-

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ture of ciliate communities was determined foremost by the host taxonomy, the peculiarities of its nutrition and social interactions (Kornilova, 2004a). Among odd-toed ungulates, the species diversity of endosymbionts has been studied mainly in representatives of the genus *Equus*, primarily in domestic horses (Strelkow, 1939; Ito et al., 1996; Gürelli, 2012, 2023; Gürelli and Göçmen, 2010; Gürelli et al., 2015, 2019; Kornilova et al., 2019). The fauna of equine endosymbiotic ciliates includes more than 80 species, some of which are found in other ungulates, and some are specific to this group of hosts (Cedrola et al., 2019). Among members of the genus *Equus,* the species composition of endobionts is rather uniform. Some species of ciliates, however, have only been found in a particular host species, such as kulans and zebras (Kornilova et al., 2021, 2023). Thus, it can be assumed that there is a certain specificity in the structure of ciliate communities in different host species.

The mechanisms of formation of endosymbiotic ciliate communities in the intestines of odd-toed ungulates, and equids in particular, remain poorly understood. To learn the influence of various factors on the structure of such communities, it is of considerable interest to study the diversity of Przewalski's horse endosymbionts. This equine species has completely disappeared from its natural habitats; the last time wild Przewalski's horses were seen in 1968 (Turghan et al., 2022). All Przewalski's horses that exist today are descendants of 11 individuals from zoos and one domestic horse (Klimov, 2018). Thus, due to the almost complete extinction of the hosts and the loss of their natural habitat, the community of endobiotic ciliates of the Przewalski's horse has actually been formed de novo since the horses' breeding in European zoos started in the early 20th century.

Currently, the number of Przewalski's horses is gradually increasing, and projects for their reintroduction into natural habitats are being successfully implemented. In 2015, a one of such projects was launched in our country in the Orenburg Nature Reserve (Tyurin, 2019). As part of the international project for the conservation of Przewalski's horses, an international studbook has been created, which keeps track of all remaining horses' lineages (Turghan et al., 2022). This allows the optimization of crossbreeding within a population and the reduction of inbreeding, as well as the tracing of the life history of different individuals over a number of generations. The analysis of the available data, therefore, makes it possible to investigate the influence of various factors on the communities of endobiotic ciliates, such as the founder effect or the food supply.

We investigated the species diversity of endobiotic ciliates in Przewalski's horses from the Orenburg Nature Reserve for the first time and analyzed the obtained data on the hosts' lineages and habitats. This is the first attempt to analyze the influence of these factors on the structure of endobiont ciliate communities in a given host. In addition, a comparative analysis of the species composition of endobionts in Przewalski's horses and domestic horses living in the same area was conducted.

Material and methods

The Przhewalski's Horse Reintroduction Center in the Orenburg Nature Reserve is located in the "Preduralskaya Steppe" area at the junction of the Belyaevsky and Akbulak districts of the Orenburg Region. The territory of the reintroduction center is represented by the sites of typical feather-grass steppe with hilly landscapes. The first Przewalski's horses were brought to Orenburg Region from the French semi-reserve Le Villars in 2015. The next import of horses – from the Hungarian Hortobády National Park – took place a year later. Currently, there are 108 horses living on the territory of the Orenburg Nature Reserve, some of which were born here.

The population has a social structure characteristic of this species: it consists of harem and bachelor groups. Females and immature males form a harem, headed by a sexually mature male. The change of the harem stallion in this territory occurs once every 2-3 years, and this entails significant changes in the composition of the harem. In addition to harems and bachelor groups, several individuals live alone. Social groups live in a certain area for no more than 4-6 months; after that, the horses look for a new grazing area. In winter, horses are fed hay once a week.

In addition to the free-ranging horses, several horses are kept in two corrals for two and a half years in order to study the species' behavior and conduct excursions (Tyurin, 2019). The area of each acclimatization paddock is 45 hectares, a small bachelor group (4 individuals) lives in the first paddock and a mare with a stallion in the second. In the paddocks, horses are fed much more frequently:

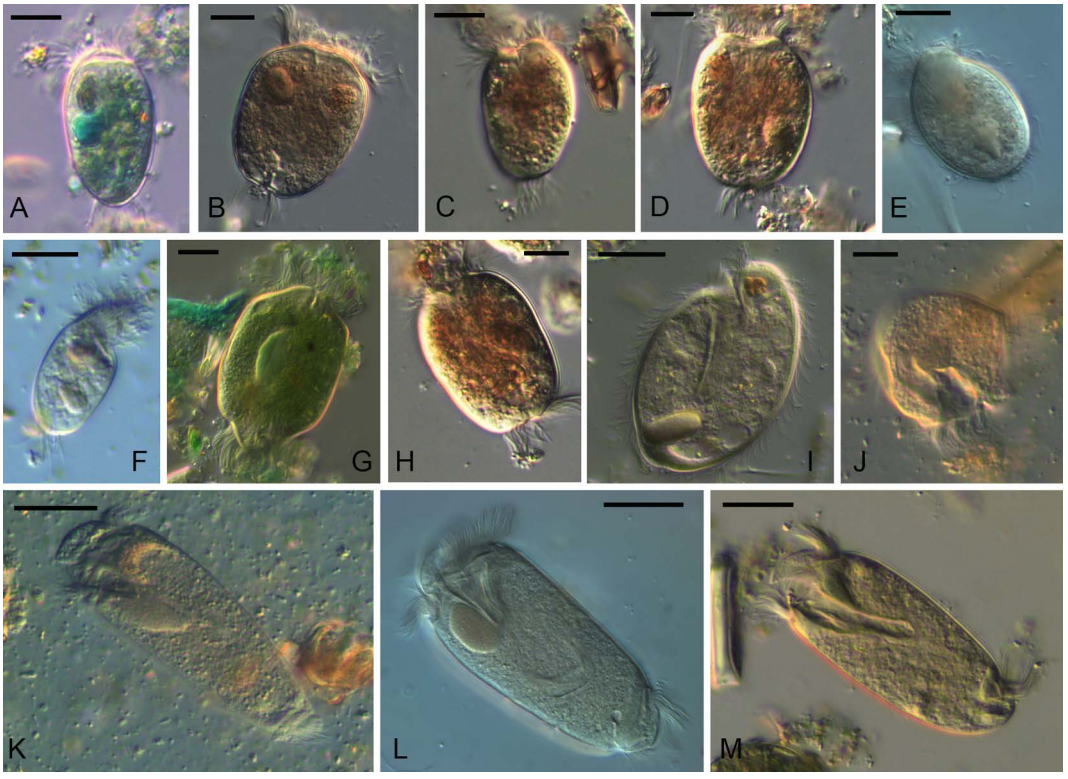

Fig. 1. Ciliates from fecal samples of Przewalski's horses from Orenburg Nature Reserve; representatives of families Buetschliidae (A–H), Paraisotrichidae (I), Blepharocorythidae (K–M). A – *Bundleia dolichosoma*, B – *B. postciliata*, C – *B. nana*, D – *B. inflata*, E – *Holophryoides macrotricha*, F – *Polymorphella ampulla*, G – *Didesmis quadrata*, H – *Fiorentinus ovalis*, I – *Paraisotricha colpoidea*, J – *Circodinium minimum*, K – *Blepharocorys angusta*, L – *B. curvigula*, M – *B. microcorys*. Scale bars: A–H, J – 10 μm, I, K–M – 20 μm.

twice a day, 4 days a week during the tourist season (from May to September); in winter, once every two days.

For this study, fecal samples of *Equus ferus przewalskii* (8 females, 6 males) were collected in August 2024 in the "Preduralskaya Steppe" area of the Orenburg Nature Reserve, as well as 2 fecal samples – from *Equus caballus* females in the village of Karpovka, located in the same area. According to available data, contacts between Przewalski's horses from the reserve and domestic horses were practically excluded.

Samples were fixed in 8% formaldehyde, in ratio 1:1 (formaldehyde solution:sample), and kept at room temperature in the dark. Ciliate species were identified and photographed using a Leica DM2500 microscope (Leica Microsystems, Germany) equipped with Nomarsky differential interference contrast and a Leica DFC495 (8.0MP) digital camera. Nuclei were stained by 1% methyl green solution. Species identification followed Strelkow (1939) and Kornilova (2003). Primary processing of data on species composition was performed in Excel. The number of ciliates of different species in *Equus ferus przewalskii* fecal samples was counted using the calibrated drop method (drop volume is 100 μl) in at least three replicates (Kornilova, 2004b). For rare species, the number of replicates was increased, sometimes up to 5. During the analysis, the number of individuals per 100 μl of the sample (in 3–5 replicates) of certain species of ciliates was assessed.

To visualize differences in ciliate species composition among horse groups, non-metric multidimensional scaling (nMDS; Shepard, 1962) was performed using the metaMDS() function from the Vegan package (Oksanen et al., 2026) based on Bray-Curtis's dissimilarity matrix. Group centroids were labeled by calculating the mean coordinates from the resulting ordination. To assess the degree of between-group differences, the R-statistic from ANOSIM (Analysis of Similarities; Clarke, 1993) was computed. This value was used solely to evaluate the magnitude of group separation, without testing its statistical significance. To test the hypothesis regarding differences in ciliate community composition across three factors (sex,

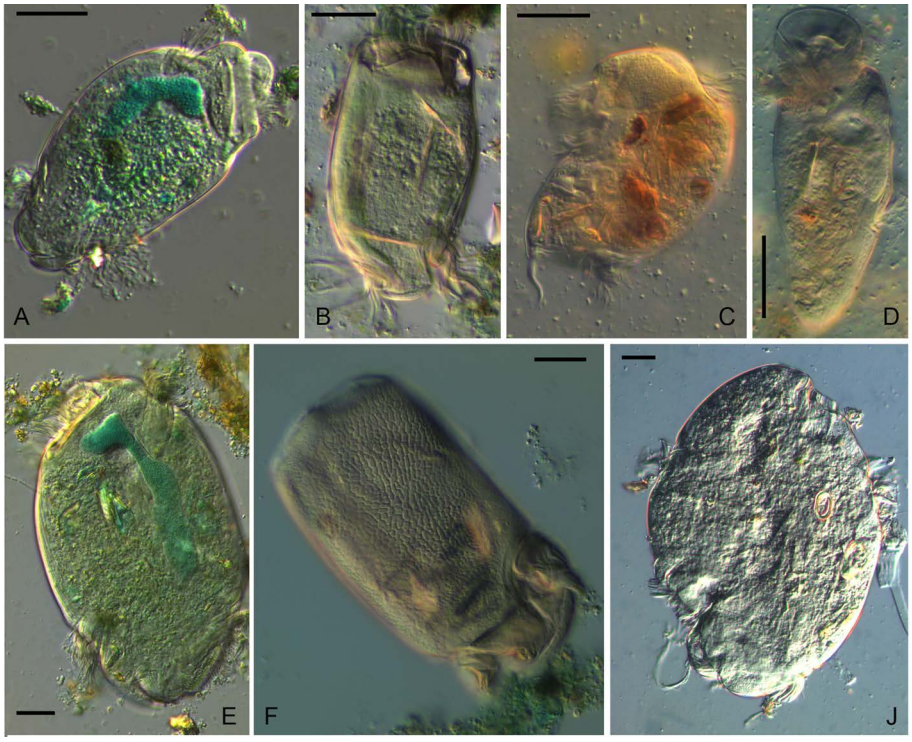

Fig. 2. Ciliates from fecal samples of Przewalski's horses from Orenburg Nature Reserve; representatives of families Spirodiniidae (A, C–E, J) and Cycloposthiidae (B, F). A – *Tetratoxum parvum*, B – *Cycloposthium bipalmatum*, C – *Triadinium caudatum*, D – *Spirodinium confusum*, E – *T. unifasciculatum*, F – *C. edentatum*, J – *Cochliatoxum periachtum*. Scale bars: 20 μm.

management conditions, and origin of Przhevalski's horses), PERMANOVA (Permutational Multivariate Analysis of Variance; Anderson, 2001) was employed. P-values were estimated using permutation tests (9,999 permutations). The PERMANOVA model included only main effects, without factor interactions. Subsequent pairwise group comparisons for the factor "origin" (three levels) were conducted using PERMANOVA, with Bonferroni correction applied to the p-values.

All calculations were performed using the adonis2() function from the Vegan package. To assess the contribution of ciliate species to differences between groups, Similarity Percentage (SIMPER) analysis was employed (Clarke, 1993). The analysis was performed with simper() function from the vegan package.

Results and discussion

In fecal samples of Przewalski's horses from the Orenburg Nature Reserve, 20 species of ciliates belonging to 11 genera and 5 families were found (Figs 1, 2). All identified ciliate species were typical for equids (Cedrola et al., 2019). It is worth noting that Przewalski's horses from other bioreserves and zoos also did not have any specific endobionts (Kornilova et al., 2023). It is known that ciliates specific to a particular species of the genus *Equus* are found in natural habitats and disappear from the ciliate communities when the host is kept in captivity (Kornilova et al., 2024). Thus, the absence of specific types of endobionts in modern Przewalski's horses is in good agreement with the history of the derivation of their ciliate fauna. Even if Przewalski's wild horses had specific types of ciliates, they could have been lost during long-term captivity.

The species composition of endobionts of Przewalski's horses and domestic horses from the village of Karpovka differed significantly (Figs 3, 4). For example, in fecal samples of domestic horses, unlike Przewalski's horses, *Tripalmaria dogieli, Spirodinium equi, Ditoxum funinucleum*, and *Allantosoma intestinale* were found. At the same time, *Cycloposthium bipalmatum, Holophryoides macrotricha, Polymorphella ampulla*, and *Fiorentinus ovalis*, which were quite numerous in Przewalski's hor-

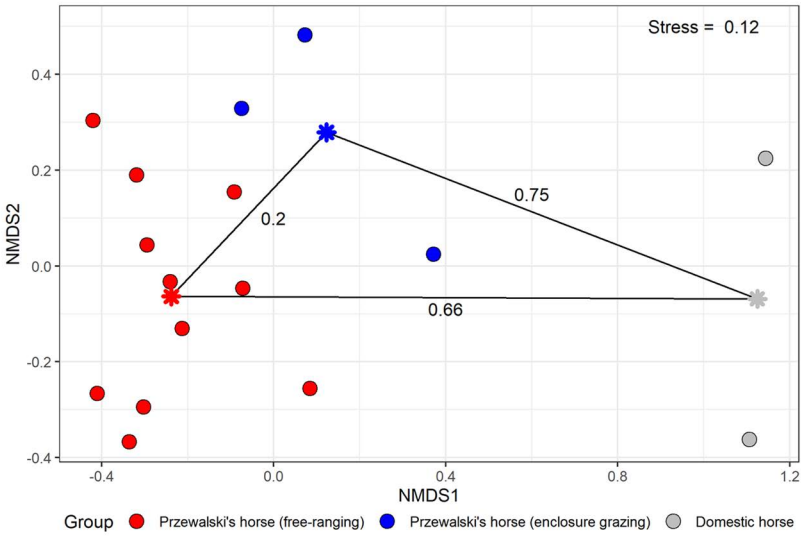

Fig. 3. Non-metric multidimensional scaling (nMDS) ordination of samples from Przewalski's horse (free-ranging and enclosure populations) and domestic horse groups. Group centroids and between-group dissimilarities (ANOSIM R-statistic) are shown.

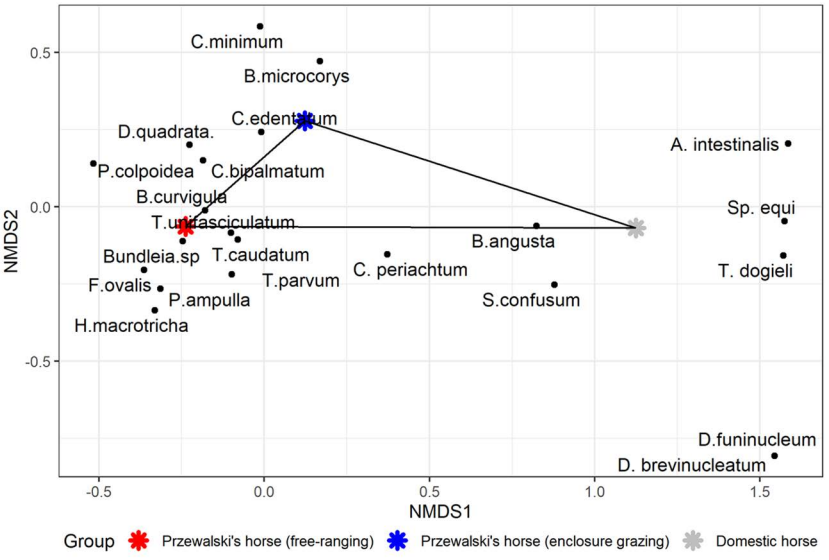

Fig. 4. Species ordination in nMDS axes consistent with Fig. 3.

ses, were completely absent in domestic horses. Such differences in the structure of endobiotic ciliate communities may be associated with the lack of social contacts between hosts, as well as differences in their diet.

The degree of dissimilarity (ANOSIM R-statistic) between free-ranging and captive Przewalski's horses was noticeably lower (low R value) than the similarity of either of these groups to domestic horses. The PERMANOVA procedure revealed a statistically significant effect of the keeping type of Przewalski's horses on the ciliate community and a marginally significant effect of horse origin (Table 1).

However, pairwise post-hoc comparisons of the groups corresponding to the 'Origin' factor, adjusted for multiple comparisons, did not reveal any reliable significant differences between the groups. The lack of strong differences between horses of different origins was supported by the ordination (Fig. 5), which did not show distinct clusters of points corresponding to the different origin groups. This fact can be explained by the population structure of the animals. The exchange of ciliates between

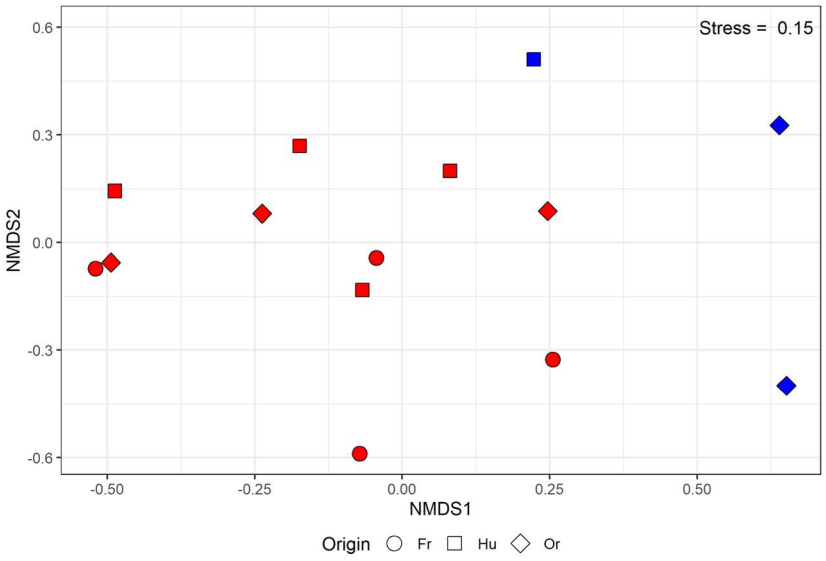

Fig. 5. Non-metric multidimensional scaling (nMDS) ordination of samples from Przewalski's horse with varying origins and keeping conditions (red – free grazing, blue – enclosure grazing).

horses occurs primarily through coprophagy. The composition of horses in harems changes every few years, which ensures an even distribution of ciliates throughout the entire population. However, it should be noted that coprophagy is characteristic mainly of young foals; in adult horses on free grazing, this phenomenon is rare. During long-term observations of the behavior of Przewalski's horses in the Orenburg Reserve, no cases of coprophagia were noted. It can be assumed that the similarity in the species composition of endobiotic ciliates may be associated with the physiological and ecological features of Przewalski's horses.

Below, we consider only the effect of the keeping type factor. The application of the SIMPER procedure showed that the greatest contribution (cumulative contribution to dissimilarity > 0.6) to the dissimilarity between free-ranging Przewalski's horses and individuals kept in enclosures was made by three ciliate taxa: *Bundleia* sp., *P. ampulla*, and *H. macrotricha* (Table 2). All three taxa had an order of magnitude higher abundance in samples from free-ranging horses compared to animals grazing in enclosures. *Bundleia* sp., *P. ampulla*, as well as *H. macrotricha* are bacterivorous ciliates. Therefore, high abundance of these endobionts may correlate with an enrichment of intestinal microflora due to a more diverse host diet. Indeed, free-range horses primarily feed on grass, especially turf and rhizome grasses, while horses grazing in enclosures were fed primarily oats and hay. In addition, the exchange of endobiotic ciliates between individuals in the population through coprophagy was limited in corrals. The total number of endobionts in fecal samples can also be influenced by the level of physical activity of animals.

Table 1. Results of PERMANOVA for comparisons of groups of horses depending on the effect of different factors.

Term	Df	SS	R^2	F-statistic	p-value
Sex	1	0.23	0.07	1.28	0.2672
Type	1	0.70	0.22	3.92	0.0103
Origin	2	0.81	0.25	2.26	0.0439
Residual	9	1.61	0.51		
Total	13	3.18	1.00		

At the same time, no significant differences were found in the Shannon diversity indices of the ciliate communities between free-ranging individuals (mean H = 1.9) and individuals from enclosures (mean H = 2.0) (t = 0.6, p = 0.60). Thus, the keeping type does not have a significant effect on the structure of the ciliate community but strongly influences the abundance of some individual species.

We can conclude that the communities of endobiotic ciliates of Przewalski's horses from the Orenburg Reserve are quite uniform in their species composition but differ significantly from the communities of endobionts of domestic horses living at the same territory. The most obvious explanation for such differences is the isolation of animals and differences in nutrition. However, we can assume that the uniformity of the species composition of

Table 2. Mean species abundance (number of individuals) in samples from free-ranging and enclosure grazing Przewalski's horse and cumulative contribution to between-group dissimilarity (SIMPER analysis) (the standard deviation is given in brackets).

Species	Free	Enclosure	Cumulative Contribution
Bundleia sp.	24.78 (30.78)	3.33 (1.76)	0.28
Polymorphella ampulla	17.85 (21.69)	1.22 (1.35)	0.47
Holophryoides macrotricha	12.7 (13.79)	0.22 (0.39)	0.62
Blepharocorys curvigula	4.36 (3.77)	3.33 (4.1)	0.71
Fiorentinus ovalis	4.05 (4.71)	0.08 (0.14)	0.76
Didesmis quadrata.	1.63 (1.94)	0.53 (0.71)	0.80
Blepharocorys microcorys	0.58 (1.25)	1.34 (1.15)	0.84
Cycloposthium edentatum	0.72 (0.7)	1.64 (2.05)	0.88
Cycloposthium bipalmatum	1.07 (1.71)	1.2 (1.29)	0.91
Triadinium caudatum	2.18 (2.77)	0.28 (0.05)	0.93
Tetratoxum unifasciculatum	1.2 (1.68)	0.42 (0.52)	0.95
Tetratoxum parvum	1.21 (1.23)	0.19 (0.17)	0.97
Circodinium minimum	0.14 (0.4)	0.33 (0.34)	0.98
Cochliatoxum periachtum	0.06 (0.13)	0.33 (0.58)	0.99
Spirodinium confusum	0.02 (0.08)	0.08 (0.14)	0.99
Paraisotricha colpoidea	0.11 (0.13)	—	1.00
Blepharocorys angusta	0.02 (0.08)	0.08 (0.14)	1.00

ciliates in Przewalski's horses reflects a certain degree of specificity in the interactions of endobionts with the host. Meanwhile, to confirm this hypothesis, further studies of the fauna of endobiotic ciliates in horses from other natural reserves are necessary.

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