

Evidence for Local Ciliate Endemism in an Alpine Anoxic Lake

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Abstract

Despite its long history, biogeography has received relatively little attention within the field of microbial ecology. Consequently, a fierce debate rages whether protists inhabit restricted geographic areas (endemism hypothesis) or are globally dispersed (ubiquitous dispersal hypothesis). The data presented in this article support the endemism hypothesis. We succeeded in isolating an oligohymenophorean ciliate from a microbial mat in a meromictic anoxic alpine lake (Alatsee) in Germany. The ciliary pattern and the morphometry of this isolate are remarkably similar to *Urocentrum turbo* (Mueller, 1786) Nitzsch, 1827. However, the organism does not possess trichocysts, a conspicuous and characteristic feature of *U. turbo*. Instead, the *U. turbo*-like isolate from lake Alatsee displays merely trichocyst Anlagen (“ghosts”) in the cytoplasm that are only visible after protargol impregnation and which become never attached to the cell’s cortex. Despite the distinctness of this difference, such a morphospecies has not been described from any other environment. Thus, we suggest that the *U. turbo*-like isolate from lake Alatsee is a local endemic ecotype, although the sequences of the 18S rRNA, ITS1, 5.8S rRNA, and ITS2 genes are nearly identical to those of *U. turbo* (Mueller, 1786) Nitzsch, 1827. This indicates that neither 18S rDNA nor ITS1, ITS2, and 5.8S rDNA sequences are reliable means to conclusively resolve different morphospecies or ecotypes of ciliates. As a consequence, we argue that protist species richness can only be reliably accounted for by considering both molecular and morphological data.

Introduction

Protists are mostly unicellular eukaryotes that are an essential component of microbial food webs [44] and play key roles in global biogeochemical cycles [49]. They are structurally and metabolically a highly diverse group of organisms and include autotrophs, heterotrophs, and mixotrophs and occur in virtually all habitats. Considering the obvious importance of protists in our planet’s ecosystems, it is remarkable that even experts in the field cannot agree whether these protists exhibit biogeographic patterns. Biogeography is the observation, recording, and explanation of the geographic ranges of organisms [41]. The interest of studying the biogeography of protists is the elucidation of biological and physical factors controlling the presence or absence of species. This area of study aims to reveal where organisms live, at what abundance, and why, and offers insights into the mechanisms that generate and maintain diversity, such as speciation, extinction, and species interactions [8, 34].

Despite biogeography’s long and distinguished history dating back to the pre-Darwinian period [34], this field of research has received surprisingly little attention in microbial ecology [8, 9]. Current ideas on protist biogeography fall into one of two schools: one assumes a ubiquitous dispersal for all species (“ubiquitous dispersal hypothesis” [11, 12]), whereas the other allows for at least some endemism (“moderate endemism hypothesis” [15, 18]). The first assumption is based on a conceptual prejudice dating back to the previous century [2, 4]: essentially, biogeography does not exist as microorganisms are easily dispersed, i.e., “all microbes are everywhere and the environment selects” [11]. This became an elementary paradigm in microbial ecology. Some fundamentals of this hypothesis are a high gene flow, a high rate of migration, and a low rate of allopatric speciation [10]. As a consequence, estimates of free-living protozoa species are low (<20,000). Most of those, it is commonly assumed, already have been documented [11].

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The endemism hypothesis is based on the discovery of many conspicuous ciliates (“flagship species”) in certain specific geographic regions despite intensive sampling efforts [14, 16, 17, 22] elsewhere. This hypothesis is also supported by molecular studies, which have identified genetically distinct forms of morphologically identical species (cryptic species, [25, 30, 31, 53, 54]). The assumption of endemic species favors a low gene flow, a local radiation, and many protists yet to be discovered [30]. It maintains that a single phylum of protozoa, the ciliates, possibly comprises more than 30,000 species [15]. Although sequence data on ciliate diversity is generally scarce, the few molecular studies conducted thus far on environmental samples or isolated organisms seem to support the latter hypothesis (e.g., [1, 36, 39, 47, 53]).

The identification of endemic microbes may be a major step in addressing basic questions not only in microbial ecology but also in evolution. Such information could explain what enables a species to live where it does; what may prevent it from colonizing other areas; how a species comes to be confined to its present range, which are a species’ closest relatives, and where they can be found; where its ancestors lived; and how climatic changes may have shaped species distribution. These questions are independent of the scale of spatial patterns, whether it be global, local, or on a microscale. In this article, we introduce a newly discovered ciliate morpho-species (ecotype) isolated from a remote anoxic alpine lake. Based on the criteria discussed in this article, this isolate can be characterized as a neoendemic protist ecotype.

Methods

We isolated the *Urocentrum turbo*-like ciliate from the meromictic alpine lake Alatsee, Allgaeu, Germany in July 2005. The 18-ha large and 35-m deep lake is of postglacial origin and is characterized by a chemocline at 15–18 m (Fig. 1), which is caused by the lake’s geography (ca. 8,000–10,000 years old, [23, 55]). At the chemocline, purple sulfur bacteria form a 1-m thick reddish layer in the water column. As this layer may at times extend to the lake’s surface, lake Alatsee is also called the “bleeding lake.” More details about the sampling site are available at (http://web.mac.com/stoeck_lab/iWeb/Lab%20Homepage/Sampling%20Sites/Sampling%20Sites.html).

Undisturbed samples of microbial mats were taken from the bottom of the lake below the chemocline by scuba diving. Therefore, we used special sampling devices, which could be sealed under water. The samples were kept for several hours at approximate ambient bottom water temperature (4–6°C; see Fig. 1) until isolation of the organism in the laboratory. We isolated

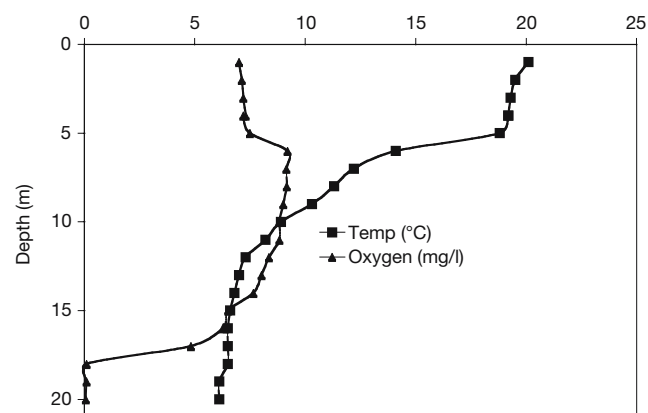


Figure 1. Oxygen and temperature profiles in lake Alatsee at the time of sampling.

the target organism by collecting individual morphologically conspicuous cells from lake Alatsee enrichment cultures (site water enriched with some squashed wheat grains). Nonclonal pure cultures of the target ciliate were established by growing the isolate at 4°C in sterile-filtered lake Alatsee water enriched with squashed wheat grains. For comparison, a trichocyst-bearing population of *Urocentrum turbo* (Müller, 1786) Nitzsch, 1827 was isolated from a *Sphagnum* pond near the town of Constance in Germany [32] as described above. Cells were studied *in vivo* using a high-power oil immersion objective (×100, N.A. 1.3) and differential interference contrast optics. The ciliary and nuclear patterns were revealed by protargol- and silver nitrate-impregnation [13]. The Alatsee population was cultivated for 3 months at 4°C and checked several times for the absence of trichocysts. They were absent from the beginning to the end of the investigations in three separate culture set-ups.

For analysis of 18S rDNA and ITS sequences, 10–20 individual cells each from the Lake Alatsee culture and from the Constance culture were placed into 180 µl ATL buffer (Qiagen, Hildesheim, Germany) and 20 µl Proteinase K (20 mg/ml). Subsequently, the genomic DNA of each of the strains was extracted separately using the protocol for cultured animal cells of the DNEasy Tissue kit (Qiagen) according to the manufacturer’s instructions. Amplification of the target sequences was performed via polymerase chain reaction (PCR) using the universal eukaryotic primers EukA and EukB [38] and ITS-F and ITS-R [3] covering the complete ITS1, 5.8S rDNA, and ITS2 sequences, respectively. Each PCR reaction contained 10 to 20 ng of DNA template, 2.5 U HotStar *Taq* DNA polymerase (Qiagen) in the manufacturer-provided reaction buffer (Tris-HCl [pH 8.7], KCl, and (NH₄)₂SO₄), 1.5 mM MgCl₂, 200 µM of each diethylnitrophenyl thiophosphate (dNTP), and 0.5 µM of each oligonucleotide primer. The final volume was adjusted to 50 µl with sterile water. The PCR protocol for 18S rRNA gene amplification consisted of an initial hot

start incubation (15 min at 95°C) followed by 30 identical amplification cycles: denaturing at 95°C for 45 s, annealing at 55°C (18S rDNA)/53°C (ITS) for 1 min, extension at 72°C for 2.5 min and final extension at 72°C for 7 min. Negative control reactions included bacterial (*Escherichia coli*) DNA as a template. The resulting PCR products were cleaned (PCR MinElute Kit, Qiagen) and cloned into a vector (TA-Cloning kit, Invitrogen). Plasmids were isolated (Qiaprep Spin Miniprep kit, Qiagen) from overnight cultures and PCR-reamplified using M13F and M13R primers to screen for inserts of the expected size (*ca.* 1.8 kb in case of the 18S rDNA fragment and *ca.* 500 bp in case of the ITS1-5.8S rDNA-ITS2 segment).

We wanted to assess the sequence heterogeneity between different strains (*U. turbo*-like lake Alatsee, *U. turbo* Constance, and a *U. turbo* GenBank entry) compared to the intrastain heterogeneity. To account for multiple copies of the target genes within the ciliates, we randomly selected three positively screened clones that carried the complete ITS1, 5.8S rRNA, and ITS2 genes of each, the Alatsee strain as well as the Constance strain, for bidirectional sequencing. ($n_{\text{total}}=6$). Similarly, we selected two positively screened representative clones for each strain carrying the amplified 18S rDNA fragment. The sequencing reactions applied standard

M13F and M13R primers and the internal primer E528 [47] and were performed using an Applied Biosystems (ABI) 3730 DNA Stretch Sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. As a result of bidirectional sequencing, we obtained a twofold coverage of each gene and clone. Before sequence assembly, we performed a Phred20 processing of the sequences using the Program CodonCode Aligner (CodonCode corporation, Dedham, MA, USA) to ensure high-quality sequences.

We aligned all sequences together with a GenBank *U. turbo* 18S rDNA sequence (accession number AF255357) using ClustalX [52]. Sequence similarities were calculated based on pairwise comparisons using the PAUP software package 4.0b10 [51].

Results

Brief description of the *Urocentrum turbo*-like ciliate from lake Alatsee (Fig. 2a–f; Table 1). The size of the ciliate isolated from lake Alatsee is 53–80×42–72 µm, with an arithmetic mean of 65×57 µm (Table 1). The cell is barrel-shaped with broadly rounded ends and slightly waisted equatorially, narrowing slightly posteriorly (Fig. 2a, e). The equatorial oral aperture is located at the anterior end of a longitudinal groove situated in the

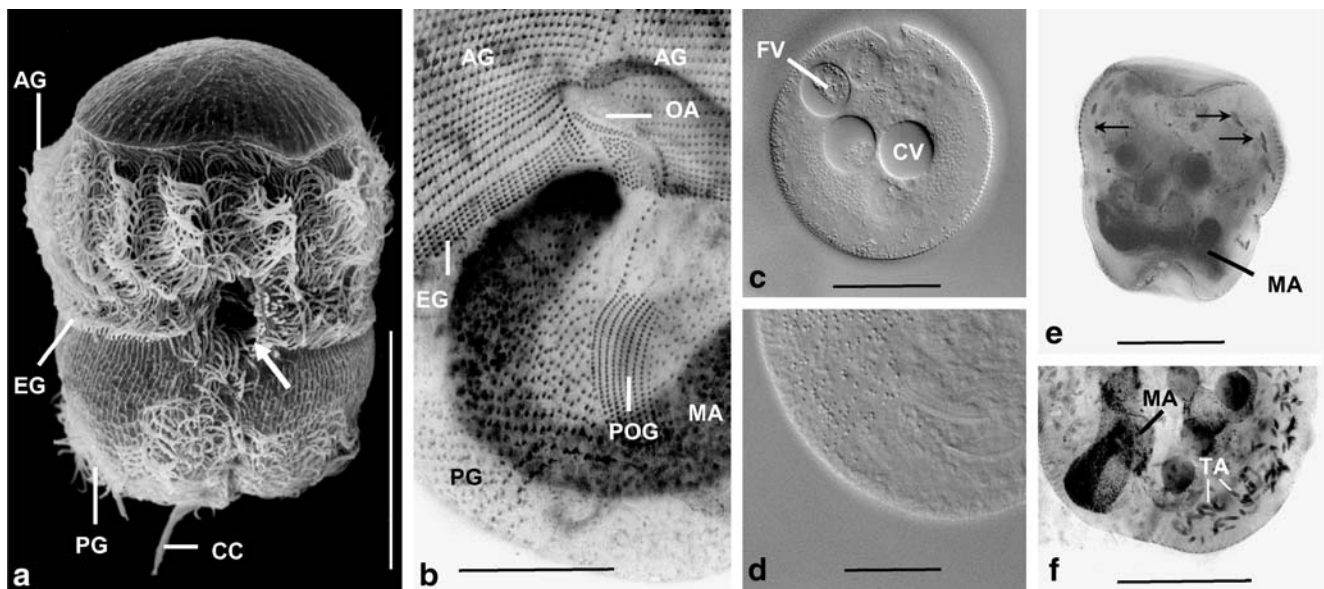


Figure 2. *Urocentrum turbo* (a) and *Urocentrum turbo*-like isolate from lake Alatsee (b–f) in the scanning electron microscope (a), after protargol impregnation (b, e, f), and from life (c, d). (a) Ventral view showing the anterior, equatorial and posterior ciliary girdle. Arrow points to the oral aperture; (b) the ciliary pattern is the same as in *Urocentrum turbo* (Mueller, 1786) Nitzsch, 1827 (compare Fig. 3). (c, e) In contrast to *Urocentrum turbo* (Mueller, 1786) Nitzsch, 1827, no extrusomes are visible in live observation (compare to Fig. 3d); (e) ventral view of a protargol-impregnated cell, in contrast to *Urocentrum turbo* (Mueller, 1786) Nitzsch, 1827, no extrusomes are visible that are attached to the cortex; arrows mark trichocyst anlagen in the cytoplasm (compare to Fig. 3b and c); (f) protargol-impregnation visualizing trichocyst anlagen (ghosts) in the cytoplasm that are not visible in vivo (c, d) and that do not get attached to the cell's cortex as in *Urocentrum turbo* (Mueller, 1786) Nitzsch, 1827 (see Fig. 3b and c). AG = Anterior girdle, CC = caudal cirrus, CV = contractile vacuole, EG = equatorial girdle, FV = food vacuole, MA = macronucleus, MI = micronucleus, OA = oral apparatus, PG = posterior girdle, TA = trichocyst anlagen (ghosts), POG = postoral groove. Scale bars 10 µm (b, d) and 30 µm (a, c, e, f).

Table 1. Morphometric data on *Urocentrum turbo* (upper line) and the *Urocentrum turbo*-like ciliate from Lake Alatsee (lower line)

Characteristics ^a	<i>x</i>	<i>M</i>	<i>SD</i>	<i>SE</i>	<i>CV</i>	<i>Min</i>	<i>Max</i>	<i>n</i>
Body, length	47.4	48.0	4.6	1.3	9.7	40.0	55.0	13
	65.2	65.0	7.2	2.0	11.0	53.0	80.0	13
Body, width	36.9	36.0	3.2	0.9	8.7	32.0	44.0	13
	57.6	57.0	9.3	2.6	16.2	42.0	72.0	13
Body length/width, ratio	1.3	1.3	0.2	0.1	13.0	1.1	2.1	13
	1.2	1.1	0.2	0.1	17.8	1.0	1.7	13
Anterior body margin to upper margin of mouth entrance	16.3	16.0	2.1	0.6	12.9	13.0	20.0	13
	27.3	28.0	5.1	1.4	18.8	16.0	37.0	13
Anterior body margin to midregion of macronucleus	32.3	32.0	4.6	1.3	14.2	25.0	39.0	13
	42.6	45.0	5.4	1.5	12.6	35.0	50.0	13
Anterior body margin to posterior margin of middle ciliary girdle	32.8	33.0	3.9	1.1	12.0	26.0	38.0	13
	40.5	40.0	4.9	1.4	12.2	30.0	47.0	13
Adoral membranelle 1, length	21.3	21.0	1.0	0.3	4.5	20.0	23.0	13
	22.9	23.0	2.3	0.7	10.2	20.0	27.0	13
Anterior ciliary girdle, number of horizontal ciliary rows ^b	18.6	19.0	0.5	0.1	2.7	18.0	19.0	13
	18.2	18.0	0.4	0.1	2.4	18.0	19.0	13
Middle ciliary girdle, number of horizontal ciliary rows	5.0	5.0	0.0	0.0	0.0	5.0	5.0	13
	5.0	5.0	0.0	0.0	0.0	5.0	5.0	13
Postoral groove, number of ciliary rows	8.0	8.0	0.0	0.0	0.0	8.0	8.0	13
	8.0	8.0	0.0	0.0	0.0	8.0	8.0	13

CV = coefficient of variation in percent (%), M = median, Max = maximum, Min = minimum, *n* = number of specimens investigated, SD = standard deviation, SE = standard error of arithmetic mean, *x* = arithmetic mean.

^aData based on mounted, protargol-impregnated specimens. Measurements in μm .

^bAnterior dikinetid counted as one row

posterior body half. It is ellipsoidal in outline with the axis lying obliquely to the major body axis. On the right edge of the aperture is an undulating membrane adjacent to a field of stomatogenic basal bodies, and three long membranelles insert within the buccal cavity (Fig. 2a, b). The posterior oral groove contains eight ciliary rows, which form a conspicuous ciliary bundle. This bundle secretes a slimy thread on which the cell can rotate, as typical for *Urocentrum*. The somatic ciliation is restricted to three girdles. One is located around the equator consisting of short dense cilia forming five horizontal rows. Above this girdle is a much wider girdle of longer and less densely packed cilia arranged in 18–19 horizontal rows. The third girdle lies in the posterior body half and consists of long and less dense cilia that never cover

the terminal pole of the cell (Fig. 2a, b). The contractile vacuole is located in the posterior end and fed by 8–12 long canals (not shown). The macronucleus is horseshoe-shaped and located in a transverse plane of the posterior body half surrounding the micronucleus (Fig. 2b, e, f; MI not visible). All isolates from this species lack extrusomes (trichocysts) attached to the cortex (Fig. 2c–f). However, protargol-impregnation revealed trichocyst anlagen (“ghosts”) in the cell’s cytoplasm, which do not attach to the cell’s cortex (Fig. 2e, f). Also these ghosts are not visible in living specimens, not even under optimal conditions (flattened cells) and with various methods (phase contrast, interference contrast) (Fig. 2c, d).

Abundance of this species in the microbial mat sample was around 100 cells/mL, indicating that it forms

Table 2. 18S rDNA sequence similarity (%) between *Urocentrum turbo* (GenBank accession number AF255357), two *U. turbo* clones collected from a *Sphagnum* pond near Constance, Germany (30), and two clones of the *U. turbo*-like isolate from the anoxic lake Alatsee

18S rDNA sequence similarity (%; 1750 bp)				
	<i>U. turbo</i> (GenBank)	<i>U. turbo</i> Constance clone 1	<i>U. turbo</i> Constance clone 2	<i>U. turbo</i> -like Alatsee clone 1 <i>U. turbo</i> -like Alatsee clone 2
<i>U. turbo</i> (GenBank)				
<i>U. turbo</i> Constance clone 1	99.71			
<i>U. turbo</i> Constance clone 2	99.77	99.71		
<i>U. turbo</i> -like Alatsee clone 1	99.65	99.60	99.65	
<i>U. turbo</i> -like Alatsee clone 2	99.60	99.54	99.60	99.48

The matrix is based on pairwise comparisons of 1750 aligned base pairs (bp). The GenBank sequence was retrieved from an organism isolated from Lake Guelph, Canada (48).

Table 3. Sequence similarity (%) between three individual randomly sequenced ITS1-5.8S rDNA-ITS2 segments each from *U. turbo* collected from a *Sphagnum* pond near Constance, Germany (30) and from the *U. turbo*-like isolate collected from below the chemocline of lake Alatsee

	ITS1-5.8S rDNA-ITS2 segment sequence similarity (%; 484 bp)					
	<i>U. turbo</i> Constance clone 1	<i>U. turbo</i> Constance clone 2	<i>U. turbo</i> Constance clone 3	<i>U. turbo</i> -like Alatsee clone 1	<i>U. turbo</i> -like Alatsee clone 2	<i>U. turbo</i> -like Alatsee clone 3
<i>U. turbo</i> Constance clone 1						
<i>U. turbo</i> Constance clone 2	99.60					
<i>U. turbo</i> Constance clone 3	99.60	100				
<i>U. turbo</i> -like Alatsee clone 1	99.60	100	100			
<i>U. turbo</i> -like Alatsee clone 2	99.60	100	100	100		
<i>U. turbo</i> -like Alatsee clone 3	99.60	100	100	100	100	

The matrix is based on pairwise comparisons of 484 aligned base pairs (bp). The GenBank sequence was retrieved from an organism isolated from Lake Guelph, Canada (48).

a stable population in lake Alatsee. Further, it grew well for some months in raw cultures enriched with some squashed wheat grains.

18S rDNA and ITS sequences. The 18S rDNA sequence of the lake Alatsee isolate is very similar to the 18S rDNA sequence of *Urocentrum turbo* from the GenBank database (accession number AF255357). We performed pairwise comparisons of the primary structures of the analyzed 18S rDNA sequences (Table 2). It is surprising to note that all 18S rDNA sequences under comparison were highly similar to each other (99.71–99.48%) with the lowest sequence similarity being between two different clones of the same strain (culture). In the *U. turbo*-like Alatsee clone 1, we found nine out of 1750 nucleotides that differed from the *U. turbo*-like Alatsee clone 2 (=99.48% sequence similarity). The highest sequence similarity was found between the Constance *U. turbo* strain and the *U. turbo* GenBank entry (99.71% in case of Constance clone 1 and 99.71% in case of clone 2). Thus, the 18S rDNA sequence heterogeneity within a strain (*U. turbo* lake Alatsee) exceeds the one between different strains. Consequently, based on the 18S rDNA sequence the lake Alatsee isolate is indistinguishable from the “true” *U. turbo*.

A pairwise comparison of the primary structures of the combined ITS1, 5.8S rRNA, and ITS2 genes leads to the same result (Table 3). All three individual randomly chosen and sequenced ITS1, 5.8S rRNA, and ITS2 segments of the lake Alatsee culture are identical to each other and to two ITS1-5.8S rRNA-ITS2 sequences of the *U. turbo* Constance culture. Only one of the three randomly sequenced ITS1-5.8S rRNA-ITS2 segments of the *U. turbo* Constance culture differs from all other ITS1-5.8S rRNA-ITS2 segments by two out of 484 nucleotides. Both differences are located in the ITS2 gene where an A is substituted for a G and a C is substituted for a T. Unfortunately, no intergenic spacer sequences of *U. turbo* are available from GenBank.

The sequences of this study were deposited into the GenBank database (accession numbers EF114293–EF114302) and the alignments are available from the authors upon request.

Discussion

We isolated an oligohymenophorean ciliate from a microbial mat of a meromictic anoxic alpine lake (Alatsee). The ciliary pattern (Table 1, Fig. 2b) of this isolate matches that of *Urocentrum turbo* (Mueller, 1786) Nitzsch, 1827, as reviewed by Foissner et al. [19]. Both organisms display three characteristic ciliary girdles with a highly similar and identical number of horizontal ciliary rows in the anterior and in the equatorial (middle) girdle. Furthermore, both have eight postoral groove ciliary rows. The *U. turbo*-like isolate from lake Alatsee is slightly larger (body length and width) compared to a “true” *U. turbo* population from a *Sphagnum* pond near Constance, Germany [32]. To our knowledge, no morphological data are available for the GenBank *U. turbo* isolate (Lake Guelph population, [48]). Associated with the larger size are differences in some “distance” characters, such as in the distances from the anterior body margin to the upper margin of mouth entrance and to the midregion of the macronucleus (Table 1).

Thus, only one striking difference exists, that is, the lack of functional extrusomes (trichocysts) in the lake Alatsee isolate (Fig. 2c–f compared to Fig. 3b–d). Trichocysts are a characteristic feature of *U. turbo* and easily recognizable by light microscopy even for nonciliate specialists (Fig. 3d). In fact, the trichocysts of *U. turbo* are so conspicuous and numerous that Smith [45] named the species after these organelles, *Urocentrum trichocystus*, which later [29] turned out to be a junior synonym of *U. turbo* (Mueller, 1786) Nitzsch, 1827. Typically, trichocysts attach to the cell cortex at a specific docking site between the cortical alveoli [28]. In contrast to *U. turbo* (Fig. 3b–d), this is not the case in the lake Alatsee isolate (Fig. 2c–f).

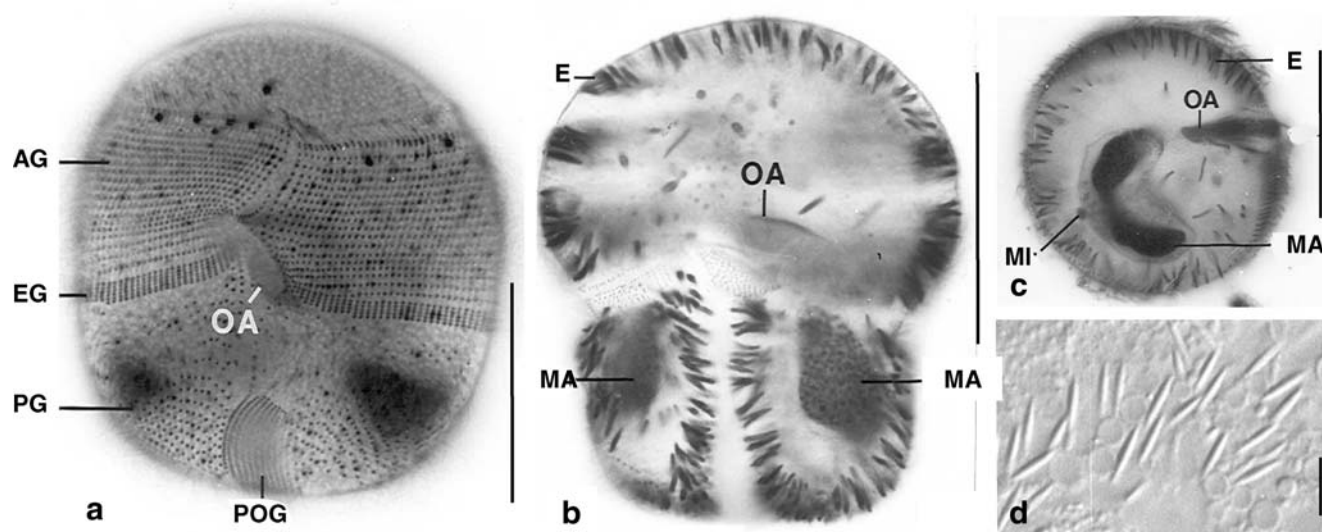


Figure 3. Protargol-impregnation (a–c) and live observation (d) of *Urocentrum turbo* (Mueller, 1786) Nitzsch, 1827. (a) Ciliary pattern of the ventral side (specimen flattened by coverslip pressure); (b) optical section showing numerous extrusomes (trichocysts) attached to the cortex; (c) optical cross section showing the nuclear apparatus and extrusomes attached to the cell's cortex; (d) high magnification of extrusomes (trichocysts) of a living specimen. AG = Anterior girdle, E = extrusomes (trichocysts), EG = equatorial girdle, MA = macronucleus, MI = micronucleus, OA = oral apparatus, PG = posterior girdle, POG = postoral groove. Scale bars 5 μ m (d), 20 μ m (c), and 30 μ m (a, b).

U. turbo is a very common ciliate often occurring in high numbers in a great variety of freshwater and even marine and brackish habitats around the globe (for review, see [19]). Because of its characteristic morphology and being the only member in the family Urocen-tridae, it is easy to identify. Indeed, there are hundreds of records from all over the world [19]. However, to date a population of this species that lacks the *U. turbo*-typical trichocysts has yet to be reported. Thus, there is good reason to assume that the isolate from lake Alatsee is a different morphospecies possibly restricted to this lake, as discussed in the following paragraph. Classifying the population from lake Alatsee as a distinct morphospecies requires a basic consideration of two questions: is the presence/absence of trichocysts a reliable character for distinguishing species, and how stable is this feature?

As regards the first question, alpha taxonomists have historically used the presence/absence of extrusomes and their shapes to distinguish species in various groups of ciliates [21, 29]. Certainly, trichocysts are a “strong” feature as their function is to defend the organism against predators [27] and, thus, dramatically influence the species’ ecology. A prime example is *Paramecium*, which invests 40% of its total protein contents in the trichocysts [42]. A lack of trichocysts could be indicative of the potential absence of predators. Although we were not able to find a described known predator of *Urocentrum*, neither in the 50-L plankton sample nor in several analyzed sediment samples, it seems unlikely that there are indeed no potential predators that feed on *Urocentrum*. This is for mainly two reasons: (1) data on

Urocentrum-predators is very scarce and very few of them are known (e.g., *Actinobolina radians*, *Dileptus margaritifer*, *Stentor coeruleus*, *Lembadion lucens*, 19), and (2) we only analyzed a minute fraction of the lakes protistan community. Because protistan communities in aquatic systems with oxygen gradients are highly diverse it would be speculative to exclude the presence of potential predators based solely upon our observations.

The second question is more difficult to address because existing data are scarce. However, *Paramecium*, a close relative of *Urocentrum*, is a fortunate exception. Although several mutations have been found in nature [40], a trichocyst-lacking population never has been described. However, it can be created in the laboratory [24]. Thus, the data available suggests that trichocysts are a reliable, strong feature to distinguish species in hymenostome ciliates.

In contrast, it is more difficult to define molecular characters that clearly distinguish two morphologically similar but not identical species from each other. In the past, sequences of the ribosomal (r)RNA have been the most commonly used marker genes in molecular taxonomic analyses (cf. [5]). Concerning the 18S rDNA sequence, it has been shown that different ciliate species may vary by only a single nucleotide [43]. Also, the 18S rDNA sequence divergence within a single ciliate species may be higher than between our lake Alatsee isolate and the “true” *U. turbo* (Table 2). For example, three different morphologically identical strains of the species *Eutintinnus pectinus* showed a mean 18S rDNA sequence divergence of 0.41% [46]. This is the same order of

magnitude as we observed of the mean 18S rDNA sequence divergence between the morphologically distinct lake Alatsee isolate and *U. turbo* (0.39%, STD = 0.04, $n = 6$). As we could observe a higher intraspecific 18S rDNA sequence heterogeneity within the lake Alatsee isolate (0.52% sequence divergence, Table 2), we can draw the following conclusion: despite a distinct morphological character that clearly distinguishes the lake Alatsee isolate from *U. turbo* (Mueller, 1786) Nitzsch, 1827, these different ecotypes are indistinguishable from each other, using the 18S rDNA sequence as a marker gene.

It is reasonable to assume that we would have found more pronounced differences between the lake Alatsee isolate and the morphologically distinct “true” *U. turbo*, analyzing the primary structure of the ITS1- 5.8S rDNA-ITS2 segment. This is because ribosomal internal transcribed spacer regions usually show a higher rate of evolution than 18S rDNA sequences and hence provide adequate polymorphism data to resolve phylogenetic relations on a genus and sometimes even on the species and population level [6, 46, 53]. Therefore, we were surprised that the ITS sequences were unable to distinguish the lake Alatsee isolate from the Constance *U. turbo*. This is particularly puzzling considering the results of other ITS sequence analysis with ciliates. For instance, van Hoek et al. [53] found more pronounced differences (>5%) in the ITS sequences of the same ciliate species (*Nyctotherus ovalis*) than we found between two morphologically distinguishable organisms (mean ITS1-5.8S rDNA-ITS2 sequence divergence between the lake Alatsee isolate and the Constance isolate runs to 0.13%, STD = 0.19%, $n = 15$). An intraspecific ITS sequence divergence exceeding the divergence observed between the lake Alatsee isolate and the Constance isolate up to 10 times was also observed within ciliate species like *Halteria grandinella* [30], *Strombidium oculatum* [30], *Cryptocaryon irritans* [7, 50], and also in other protists [29, 37].

A mean ITS1-5.8S rDNA-ITS2 sequence divergence between the lake Alatsee isolate and the Constance isolate of nearly 100% clearly indicates a common origin (ancestor) of both morphotypes. The change in a morphological character (absence of trichocysts) occurred only relatively recently, i.e., within a period of time that did not allow for distinctive changes in the ITS sequences. To date, the rate of mutation in the ITS region of ciliates has not yet been elucidated. However, such data does exist for dinoflagellates (0.75–1.33 changes per Myr, [33]), which are phylogenetically closely related to ciliates [26]. Thus, Katz et al. [30] suggest the rate of mutation in the ITS region of dinoflagellates as a rough estimate for the ITS mutation rate in ciliates. This is without doubt critical, but even if we would assume a 10-fold (or even 100-fold) higher rate of evolution in ciliate ITS compared to dinoflagellate

ITS, we conclude that a morphological speciation process will be reflected in the ITS no sooner than about 0.5–0.6 million years or 50–60,000 years after the initiation of the process. Because the lake Alatsee population cannot be older than 10,000 years as a result of the postglacial origin of the lake, the *U. turbo*-like isolate from lake Alatsee may be considered as a novel ecotype or neoendemic species [35].

In summary, our results indicate that we might have to take another variable into account that influences the global protistan species richness debate: based on sequence analysis of highly similar morphospecies [30, 31, 53, 54] and environmental molecular diversity surveys [1, 36, 39], it becomes evident that the local and probably also the global species richness is higher than estimates based on microscopic studies [11]. Our results confirm that sequences, which are highly similar to each other and, thus, may appear to belong to a single morphospecies, may in fact mask two or more different ecotypes. Such cases have been reported earlier [30, 31, 53, 54], indicating that the estimated number of protist species is much higher than currently supposed. (Pre)-alpine lakes may prove a suitable environment to study this question, as there is indeed further evidence that such lakes contain endemic ciliate species (e.g., *Ophrydium eutrophicum* Foissner), which were never detected outside the type area [20].

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