

SHORT COMMUNICATION

The Ability of *Tetrahymena utriculariae* (Ciliophora, Oligohymenophorea) to Colonize Traps of Different Species of Aquatic Carnivorous *Utricularia*

Dagmara Sirová^a , Veronika Kreidlová^b, Lubomír Adamec^c & Jaroslav Vrba^b 

a Biology Centre CAS, Institute of Hydrobiology, CZ-37005, České Budějovice, Czech Republic

b Faculty of Science, University of South Bohemia, CZ-37005, České Budějovice, Czech Republic

c Institute of Botany CAS, Dukelská 135, CZ-37982, Třeboň, Czech Republic

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Correspondence

D. Sirová, Dept. of Aquatic Microbial Ecology, Institute of Hydrobiology, Biology Centre CAS, Na Sádkách 7, 37005, České Budějovice, Czech Republic

Telephone number: +420387775872;

FAX number: +420385310248;

e-mail: dagmara.sirova@bc.cas.cz

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ABSTRACT

The host specificity of the recently described ciliate species *Tetrahymena utriculariae* was tested in a greenhouse growth experiment, which included 14 different species of aquatic *Utricularia* as potential host plants. We confirmed the high specificity of the interaction between *U. reflexa* and *T. utriculariae*, the former being the only tested host species able to maintain colonization for prolonged time periods. We conclude that this plant–microbe relationship is a unique and specialized form of digestive mutualism and the plant–microbe unit a suitable experimental system for future ecophysiological studies.

PLANT-ASSOCIATED microorganisms are now considered key drivers in ecosystem functioning and stability (Berg et al. 2014). The research of microbiota associated with aquatic plants has revealed many interesting ecological interactions and has led to the discovery of new microbial species. One such example is the complex association between aquatic carnivorous *Utricularia* plants and the microorganisms colonizing their traps (Sirová et al. 2018), the most significant of which is the newly discovered ciliate *Tetrahymena utriculariae* (Pitsch et al. 2017; Šimek et al. 2017). The aquatic carnivorous genus *Utricularia* L. (bladderwort, Lentibulariaceae) comprises about 60 rootless species (Taylor 1989). Aquatic *Utricularia* commonly grow in shallow, nutrient-poor humic waters (Adamec 2008, 2018; Guisande et al. 2007). Their shoots are mostly linear, with finely branched filamentous leaves.

The traps of aquatic *Utricularia* species are hollow, 1–6 mm large, fluid-filled bladders with a trap door (Adamec 2011; Taylor 1989). Traps can fire after mechanical stimulation or spontaneously and capture small prey or

suspended particles from the ambient water (Adamec 2018; Peroutka et al. 2008). They host a diverse community of microorganisms consisting of bacteria, ciliates, rotifers, fungi, and some algal groups (for the review, see Sirová et al. 2018), which colonize an acidic and almost anoxic trap fluid. These organisms enter young sterile traps from the environment, propagate inside, and some take part in prey decomposition. The trap turnover is usually rapid, due to the fast apical growth of *Utricularia* shoots.

The *Tetrahymena* species (Ciliophora, Oligohymenophorea) are likely the best-studied ciliates to date. However, the first green representative of the genus was discovered only recently and has raised the interest of researchers due to its highly specific lifestyle and ecophysiology (Pitsch et al. 2017; Šimek et al. 2017). *Tetrahymena utriculariae*, living in symbiosis with endosymbiotic algae (zoochlorellae) identified as *Micractinium* sp. (Chlorophyta), has been discovered in the traps of the culture-grown aquatic *Utricularia reflexa* Oliv., originating from the Okavango Delta, Botswana. Although the full life cycle of *T. utriculariae* has been documented and

some ecophysiological traits described (Šimek et al. 2017), its ecology remains virtually unexplored. It is unknown whether the current population, living under the culture conditions at the Institute of Botany CAS, Třeboň, Czech Republic, originated in Botswana or whether the *U. reflexa* plants were colonized during their growth in the culture. However, to the best of our knowledge, the ciliate has never been found outside of *U. reflexa* traps. Another intriguing question is whether *T. utriculariae* is able to colonize the traps of other aquatic *Utricularia* species and survive inside them, and how young, originally sterile, traps are colonized.

Here, we describe the outcome of a concise greenhouse growth experiment, which included 14 aquatic *Utricularia* species and was designed to test the ability of *T. utriculariae* to colonize *Utricularia* traps and survive there for extended time periods. We have also focused on testing the hypothesis that the ciliate is able to colonize new traps through a water barrier. Our aim was to shed more light on the ecology of this new, potentially important model experimental species.

MATERIALS AND METHODS

Design of the colonization experiment

On the basis of a preliminary study (S1), six *Utricularia* target species were selected for the experiment from the original set of 14. Two adult plants of *U. reflexa* (10 cm long) as a source of the ciliates were growing inside a vertically positioned porous cylinder with a diameter of 8 cm made of white plastic net (mesh size ca. 3.5 mm; see Fig. 1B). Another porous cylinder with a diameter of 12 cm was placed concentrically around the cylinder with the source plants using spacers so that it delimited a free water barrier 2 cm wide for the radial ciliate transfer. Six porous cylinders with the diameter of 6 cm were fastened radially to the wider cylinder. Each contained (depending on shoot robustness) two to three shoots (7–10 cm long) of target species: *U. aurea*, *U. gibba*, *U. purpurea*, *U. inflata*, *U. ochroleuca*, and ciliate-free *U. reflexa* (for more details, see S1). The distance between adjacent cylinders was ca. 3 cm, and the order of species was randomized (Fig. 1A). Each set of porous cylinders was placed in a plastic aquarium (volume ca. 16 liter, water depth 15 cm). Each aquarium contained 16 g (DW) of pre-soaked *Carex* litter as a substrate and humic water. Six parallel aquaria were established and floated in two containers (0.8 m²) in a naturally lit greenhouse (Fig. 1A, B). Irradiance at plant level was ca. 25% of that in the open. During the experiment, pH in all aquaria varied between 7.6 and 7.9, total alkalinity between 1.04 and 1.22 meq/liter, free CO₂ concentration ca. 40–50 µM, electrical conductivity 30.6–31.4 mS/m, and dissolved nutrient concentrations were (in µg/liter): NO₃-N, 15–43; NH₄-N, 10–18; PO₄-P, 1.0–8.4. Maximum water temperatures fluctuated between 22.5 and 33.5 °C; the difference between day and night temperatures was 4 to 6 °C. The experiment began on June 17, 2018, and was terminated after 30 days. At the end of the experiment, plants were washed under tap water and

transferred to the laboratory for estimation of *T. utriculariae* numbers in traps. The enumeration was performed in intact traps under a light microscope, and the following semi-quantitative categories were used: 0 (0 individuals/trap), 1 (1–10 ind./trap), 2 (> 10–50 ind./trap), 3 (> 50–100 ind./trap), and 4 (> 100 ind./trap). Functional traps on all shoots were evaluated.

Long-term survival experiment

To estimate whether the ciliate can survive in traps of different *Utricularia* species for prolonged time periods, two to three shoots of *U. aurea*, *U. gibba*, *U. purpurea*, *U. inflata*, and *U. ochroleuca* were put to a 3-liter mini-aquarium with a dense control culture of *U. reflexa* with the ciliate on June 30, 2018. After seven days of ciliate colonization, the plants were taken out of the mini-aquarium, thoroughly washed by tap water, and each species was inserted separately into 2.5 liter of humic water in a ciliate-free floating bottle as above. After 40 days of growth in the same 0.8 m² container in the greenhouse, the plants were washed by tap water and transferred to the laboratory for ciliate enumeration inside of the traps.

Statistical analyses

The ability of the ciliate *T. utriculariae* to colonize the traps of various *Utricularia* species through the free water barrier and the *Utricularia* species effect on the extent of the colonization were studied in an experiment with randomized complete block design (Fig. 1A, B). Mean values of semi-quantitative ciliate counts per trap were log_e(x + 1)-transformed to meet the assumptions of ANOVA. Only *Utricularia* species with nonzero means were considered for the statistical analysis. All calculations were performed using R 3.6.2 (R Core Team 2019). The effect of plant species was evaluated using ANOVA (*Utricularia* species as a fixed factor and block as a random factor) followed by a Tukey HSD post hoc test, after checking the homogeneity of variances using a Bartlett's test. The hypothesis that *U. reflexa* traps are preferentially colonized by >*T. utriculariae* was tested using planned contrasts computed in the *multcomp* 1.4–12 package in R (Bretz et al. 2020). To exclude the potential secondary transfer of ciliates from *U. reflexa* (originally free of ciliates) to the rest of investigated species, a linear regression model tested by a partial F-test was used to study the relationship between nontransformed semi-quantitative counts of ciliates and the distance from *U. reflexa* (defined as: 1—neighboring position in the box, 2—one position offset from *U. reflexa*, and 3—two positions offset from *U. reflexa*). Homoscedasticity and residuals distribution of constructed models were checked using regression diagnostic plots.

RESULTS AND DISCUSSION

The preliminary experiment (see Supporting Information) has confirmed that *T. utriculariae* individuals are capable of colonizing the traps of almost all *Utricularia* species

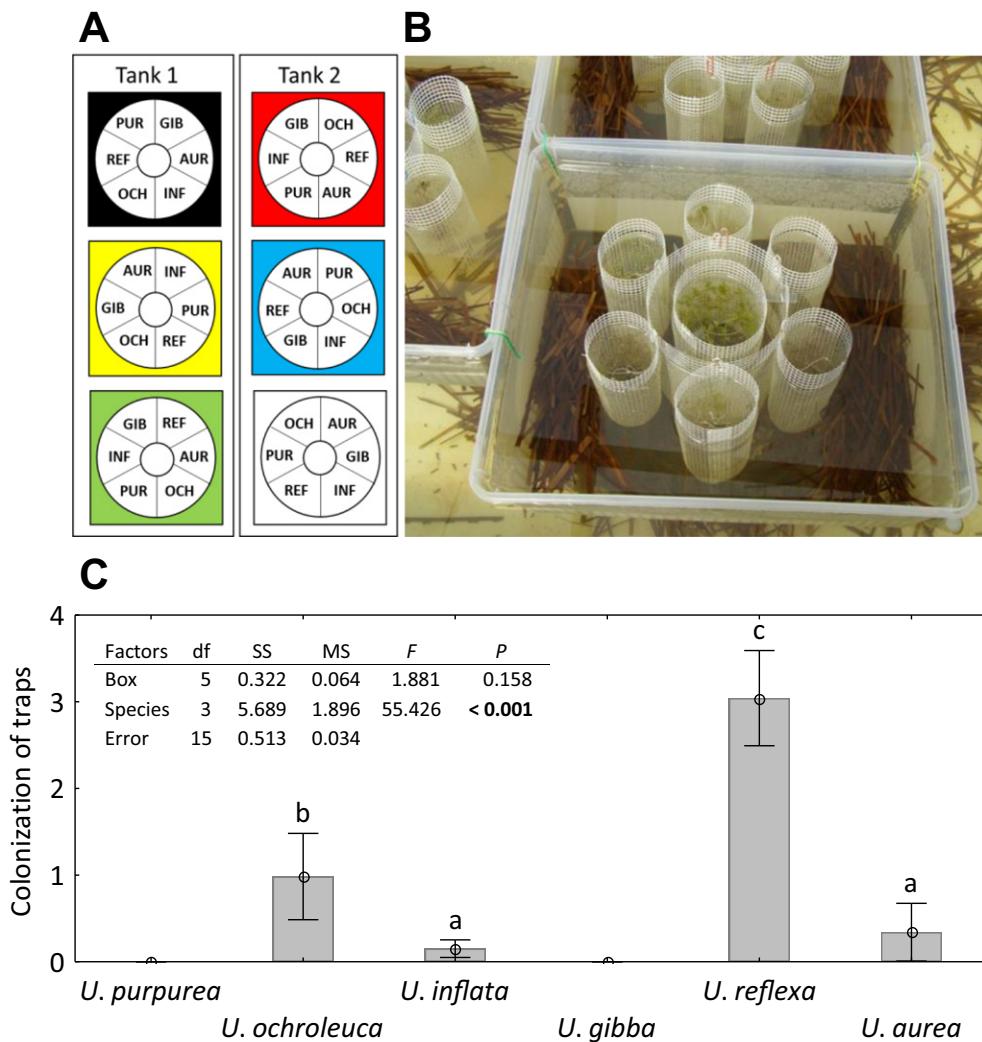


Figure 1 (A) Schematic experimental setup (randomized complete block design). The *Utricularia* species tested for *Tetrahymena utriculariae* colonization: *U. reflexa* (REF; ciliate-free), *U. aurea* (AUR), *U. gibba* (GIB), *U. inflata* (INF), *U. ochroleuca* (OCH), and *U. purpurea* (PUR). (B) Photograph of plant arrangement in the aquaria/tanks. (C) The effect of *Utricularia* species on the degree of colonization of their traps by *T. utriculariae* (semi-quantitative scale, see the Methods). Mean (column) and \pm SD (bars) are shown. The inserted table shows the results of ANOVA ($\log_e(x + 1)$ transformed data); significant differences are expressed as different letters (Tukey HSD post hoc test; $P < 0.05$).

different from *Utricularia reflexa* when their shoots are in direct contact. The main colonization experiment (Fig. 1) has shown that the colonization takes place even across 2 cm of free water barrier. This confirms that source plant and new host plant need not be in direct contact for the transfer of individual ciliates to occur. The colonization of traps by *T. utriculariae* was confirmed as species-specific ($F_{5,15} = 55.4$, $P < 0.001$; Fig. 1C). The original host, *U. reflexa*, was the most colonized species (> 100 ciliate cells per trap). On the contrary, no *T. utriculariae* individuals were found in the traps of *U. purpurea* and *U. gibba*. According to the results of the multiple comparisons, there was a significant difference between *U. reflexa* and the other *Utricularia* species tested (Tukey, $P < 0.001$). No difference was found between the colonization of *U. inflata* and *U. aurea*. Compared to these two species, a

slightly higher attractivity was observed in *U. ochroleuca*; however, overall, *T. utriculariae* significantly preferred the traps of *U. reflexa* (planned contrasts, $P < 0.001$). The degree of colonization was not affected by the proximity to *U. reflexa* in the experimental setup (partial F -test, $F_{1,16} = 0.581$, $P = 0.46$). This result, together with the fact that no *T. utriculariae* cells were found in the cultivation water or on the plant surfaces, suggests a high affinity of *T. utriculariae* for the trap lumen, possibly facilitated by some chemical cues (cf. Montagnes et al. 2008). Because of the significant differences in the colonization extent (Fig. 1), it is likely that these cues are species-specific. The colonization pattern does not seem to be related to host phylogeny or trap size, although the un-colonized *U. purpurea* belongs to a different section within the genus (Silva et al. 2018). The results of the long-term survival

experiment (not shown), where colonized plants were grown in the absence of another inoculum, show that only *U. reflexa* retains the population of *T. utriculariae* in the traps at levels of > 100 individuals per trap after 40 days of cultivation, while all the other *Utricularia* species could not provide conditions for their maintenance (0 individuals per trap after 40 days). The results show that although a short-term colonization by *T. utriculariae* of different *Utricularia* species is possible, *U. reflexa* is the optimal host, not only because of the high ciliate numbers per trap, but because these high numbers are able to persist for prolonged periods of time and continuously colonize young traps. The reasons for this may include *U. reflexa*'s trap size (largest of submerged *Utricularia* species, 6–8.5 mm long) and chemical composition of the trap fluid or different composition of plant exudates that could serve as chemical cues. As the majority of temperate *Utricularia* species form overwintering turions (Adamec 2018), there would be the need for spring re-inoculation of newly formed traps from the ambient environment in these species. The large *U. reflexa* traps are less sensitive to mechanical stimulation than those in other aquatic species (Adamec, unpubl.), which results in the specialization for trapping relatively bulky prey. High cell numbers and metabolic activity of *Tetrahymena* likely speed up the decomposition of the prey, the large mass of which could otherwise cause fouling of the trap fluid. This association can be compared to that between insect commensals and the pitchers of terrestrial carnivorous *Nepenthes* species (Bittleston 2018). Photosynthesis apparently takes place inside the traps, as the trap walls, which are mostly only two cells thick, remain transparent even under heavy pigmentation caused by the accumulation of anthocyanins (Sirová et al. 2018). The presence of endosymbiotic zoochlorellae in *T. utriculariae* is likely an important adaptation for survival under oxygen-depleted conditions (cf. Taylor and Sanders 2009). Although new *Tetrahymena* species have recently been described from traps of subtropical, mostly terrestrial *Utricularia* species, the host specificity of these ciliates is unknown and no zoochlorellae-bearing species have been reported (Cheng et al. 2019). We conclude that the relationship between *U. reflexa* and *T. utriculariae* is a unique and specialized digestive mutualism and this plant–microbe unit is a suitable experimental system for future ecophysiological studies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1. Preliminary colonization experiment.