



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**Keywords**

Affinity; ciliate; colonization; long-term survival; rootless plant; sterile traps; trap fluid; *Utricularia reflexa*; zoochlorellae.

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

Received: 13 March 2020; revised 23 April 2020; accepted April 24, 2020.
Early View publication July 10, 2020

doi:10.1111/jeu.12812

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

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.

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

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.

ACKNOWLEDGMENTS

This study was supported by the Czech long-term research development project RVO 67985939 (LA). We thank Barbora Pavlíková and Jan Pokorný for help in the greenhouse, Petr Šmilauer for his help with statistics, and Karel Šimek for critically evaluating the manuscript.

LITERATURE CITED

Adamec, L. 2008. Mineral nutrient relations in the aquatic carnivorous plant *Utricularia australis* and its investment in carnivory. *Fund. Appl. Limnol.*, 171:175–183.

- Adamec, L. 2011. The smallest but fastest: ecophysiological characteristics of traps of aquatic carnivorous *Utricularia*. *Plant Signal. Behav.*, 6:640–646.
- Adamec, L. 2018. Ecophysiology of aquatic carnivorous plants. In: Ellison, A. M. & Adamec, L. (Ed.), *Carnivorous Plants: Physiology, Ecology, and Evolution*. Oxford University Press, Oxford. p. 256–269.
- Berg, G., Grube, M., Schloter, M. & Smalla, K. 2014. Unraveling the plant microbiome: looking back and future perspectives. *Front. Microbiol.*, 5:148.
- Bittleston, L. S. 2018. Commensals of *Nepenthes* pitchers. In: Ellison, A. M. & Adamec, L. (Ed.), *Carnivorous Plants: Physiology, Ecology and Evolution*. Oxford University Press, Oxford. p. 314–332.
- Bretz, F., Hothorn, T. & Westfall, P. 2020. *Multiple Comparisons Using R*. CRC Press, Boca Raton, FL.
- Cheng, C. Y., Chang, S. L., Lin, I. T. & Lao, M. C. 2019. Abundant and diverse *Tetrahymena* species living in the bladder traps of aquatic carnivorous *Utricularia* plants. *Sci. Rep.*, 9:13669.
- Guisande, C., Granado-Lorencio, C., Andrade-Sossa, C. & Duque, S. R. 2007. Bladderworts. *Funct. Plant Sci. Biotechnol.*, 1:58–68.
- Montagnes, D. J., Barbosa, A. B., Boenigk, J., Davidson, K., Jurgens, K., Macek, M., Parry, J. D., Roberts, E. C. & Šimek, K. 2008. Selective feeding behaviour of key free-living protists: avenues for continued study. *Aquat. Microb. Ecol.*, 53:83–98.
- Peroutka, M., Adlassnig, W., Volgger, M., Lendl, T., Uri, W. G. & Lichtscheidl, I. K. 2008. *Utricularia*: a vegetarian carnivorous plant? Algae as prey of bladderwort in oligotrophic bogs. *Plant Ecol.*, 199:153–162.
- Pitsch, G., Adamec, L., Dirren, S., Nitsche, F., Šimek, K., Sirová, D. & Posch, T. 2017. The green *Tetrahymena utriculariae* n. sp. (Ciliophora, Oligohymenophorea) with its endosymbiotic algae (*Microctinium* sp.), living in the feeding traps of a carnivorous aquatic plant. *J. Eukaryot. Microbiol.*, 64:322–335.
- R Core Team. 2019. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Silva, S. R., Gibson, R., Adamec, L., Domínguez, Y. & Miranda, V. 2018. Molecular phylogeny of bladderworts: a wide approach of *Utricularia* (Lentibulariaceae) species relationships based on six plastidial and nuclear DNA sequences. *Mol. Phylogenet. Evol.*, 118:244–264.
- Šimek, K., Pitsch, G., Salcher, M. M., Sirová, D., Shabarova, T., Adamec, L. & Posch, T. 2017. Ecological traits of a zoochlorellae-bearing *Tetrahymena* sp. (Ciliophora) living in traps of the carnivorous aquatic plant *Utricularia reflexa*. *J. Eukaryot. Microbiol.*, 64:336–348.
- Sirová, D., Bárta, J., Borovec, J. & Vrba, J. 2018. The *Utricularia*-associated microbiome: composition, function, and ecology. In: Ellison, A. M. & Adamec, L. (Ed.), *Carnivorous Plants: Physiology, Ecology and Evolution*. Oxford University Press, Oxford. p. 349–358.
- Taylor, P. 1989. *The Genus Utricularia: A Taxonomic Monograph*. Kew Bulletin, Additional Series, XIV.
- Taylor, W. D. & Sanders, R. W. 2009. Protozoa. In: Thorp, J. H. & Covich, A. P. (Ed.), *Ecology and Classification of North American Freshwater Invertebrates*. Academic Press, New York, NY. p. 66.

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.