Short communication

Bacterial community associated with traps of the carnivorous plants *Utricularia hydrocarpa* and *Genlisea filiformis*

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**ABSTRACT**

The species of bacteria associated with the traps of the carnivorous plants *Utricularia hydrocarpa* Vahl and *Genlisea filiformis* A. St.-Hil. were identified by analysing 16S rRNA gene libraries. We observed larger bacterial diversity inside the traps of *U. hydrocarpa* than in *G. filiformis*. The *Clostridium* genus (Firmicutes) was the dominant group in *G. filiformis*, while *Aeromonas* (γ-Proteobacteria) and *Acidobacterium* (Acidobacteria) were the dominant genera in *U. hydrocarpa*. In general, the microbial community observed in these carnivorous plants was composed of Firmicutes (46.8%), Proteobacteria (33.9%), Acidobacteria (9.3%), Actinobacteria (4.4%), Bacteroidetes (0.8%), Chloroflexi (0.4%), Gemmatimonadetes (0.4%), Cyanobacteria (0.4%), Chlamydiae (0.4%) and Tenericutes (0.4%). Only 1.2% of the observed operational taxonomic units (OTU<sub>0.03</sub>) were shared by *U. hydrocarpa* and *G. filiformis*. The present study describes the dominant bacterial species associated with the traps of the carnivorous plant *G. filiformis* and *U. hydrocarpa* and briefly discusses the possible role of bacteria in plant prey utilisation.

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1. Introduction

The phyllosphere, defined as the area influenced by plants (Ruinen, 1961; Hardoim et al., 2008), contains different niches for highly diverse microbial populations. Therefore, the characterisation of this bacterial diversity is key to understanding not only the ecology and evolution of plant species but also their interactions with a diversity of other organisms (Lindow and Brandl, 2003; Knief et al., 2010). Among an estimated 300,000 species of angiosperms, carnivorous plants are represented by 650 species (Król et al., 2012) that display a notable convergence in their traps (usually modified leaves) and physiology. Carnivorous plants have the ability to capture, kill, digest, and use the molecular products of their prey (Juniper et al., 1989). This prey could be an important source of additional N, P, S, K and Mg, in some cases representing 50–80% of the total nitrogen mass of the plant (Adamec, 1997; Ellison and Gotelli, 2001).

Diverse microbial communities, mainly containing bacteria, algae, protozoa and rotifers, live inside the *Utricularia* and *Genlisea* traps (Skutch, 1928; Jobson and Morris, 2001; Richards, 2001; Sirová et al., 2003; Adamec, 2007; Plachno and Wołowski, 2008; Sirová et al., 2009), and could play a role in plant fitness in association with prey digestion and environmental adaptation. Sirová et al. (2003) suggested that *Utricularia* creates an environment stimulating microbial metabolism, which results in nutrient release through prey digestion similar to the plant–microbe relationship found in pitchers of *Sarracenia purpurea* L. Microbial food webs are vital components of aquatic systems, as they involve nutrient recycling by phytoplankton, bacteria and microzooplankton (Azam et al., 1983). The environment inside the *Utricularia* trap, which is completely sealed and anoxic (Adamec, 2007), contains all of the components of a complex microbial food web, indicating that the microbial community in the trap could be responsible for a significant proportion of enzymatic activity associated with prey digestion (Sirová et al., 2009). According to Adamec (2007), the maintenance of anoxic fluid inside the traps could be the mechanism for killing captured prey. An additional prediction is that the microbial community inside these traps would be adapted to this environment, which is...
characterised by limited periods of higher $O_2$ concentration after firing events followed by facultative anoxia (Adamec, 2007), allowing organisms that are more tolerant to anoxia to live commensally (Richards, 2001; Sirová et al., 2003) and contributing to prey degradation.

The role of microorganisms in the digestion process is subject to discussion in terms of the real importance to plant development. In *U. vulgaris* L., most traps have a full life cycle that is shorter than 30 days, with complete development of active traps occurring between 10 and 19 days (Friday, 1989). Therefore, trap age is considered to be vital for the ecophysiology of *Utricularia* (Friday, 1989; Sirová et al., 2003) and could reflect the microbial communities inside the vessel (Plachno et al., 2012). In these traps, Sirová et al. (2009) observed that bacteria represent 58% of the total viable microbial biomass, suggesting that they play a key role in the food web inside the trap. Although many studies have identified the bacterial species in the pitcher of the *Sarracenia* species (Peterson et al., 2008; Siragusa et al., 2007; Krieger and Kourtev, 2012a, 2012b; Koopman and Carstens, 2011; Koopman et al., 2010), no studies have described the bacterial taxa inside the traps of the *Genlisea* and *Utricularia* species. Therefore, in the present study, we describe the dominant bacterial species present inside the traps of *U. hydrocarpa* and *G. filiformis* and briefly discuss the possible role of these bacteria in plant prey utilisation.

2. Materials and methods

2.1. Plant sampling

*Utricularia hydrocarpa* (UH) and *Genlisea filiformis* (GF) traps were collected from at least five plants from natural populations at three distinct sites (cities of Cuiabá, Poconé and Santo Antônio do Leverger, Mato Grosso State, Brazil). The vouchers were deposited in the Herbarium JABU at the University of São Paulo State (Jaboticabal, SP, Brazil). The *U. hydrocarpa* plants were collected from unpolluted and clean rivers, growing as aquatic plants with other macrophytes, while the *G. filiformis* plants were collected from grassy pastures. For analysis, the traps were picked up from each plant specimen and carefully washed in sterilised water. For *U. hydrocarpa*, brown traps up to 20 cm from the growth tip, with prey debris inside were selected under stereo-epoxy microscope. A composite sample from each site, consisting of at least 100 traps per plant, was then stored at −80 °C until DNA extraction.

2.2. DNA extraction and gene libraries preparation

The traps were macerated, and total DNA was extracted using the MoBio Power Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to the supplier’s recommendations. DNA extraction and integrity were checked by agarose gel electrophoresis (0.8%, w/v) and UV visualisation using ethidium bromide.

Bacterial 16S rRNA gene fragments from the carnivorous plants UH and GF were amplified using primers 986F and 1378R (Lane et al., 1985) in a PCR mix (50 µl) containing 1 µl template DNA (4 ng), 0.2 mM dNTPs, 3.75 mM MgCl2, 0.2 µM of each primer and 2.5 U Taq DNA polymerase (Sinape Biotecnologia, São Paulo, SP, Brazil). After initial denaturation at 94 °C for 5 min, 30 cycles consisting of 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C were performed, followed by a final 10 min extension at 72 °C. Amplicons from traps collected from the same plant species at the different sites were pooled, purified using the QiAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany), and the same PCR product concentration, for different plant species were cloned into pGEM®-T easy vectors (pGEM®-T Vectors System II, Promega, Madison, WI, USA) and used to transform *Escherichia coli* DH5α competent cells. Positive colonies were picked out and used to construct two 16S rRNA gene libraries (UH and GF) with a total 500 clones. The inserts were PCR-amplified using the M13F/M13R primers, and inserts ranging from 450 to 500 bp were purified and sequenced with the 1378R primer, using the BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The 16S rRNA gene sequences were used as input data for phylogenetic and statistical analysis.

2.3. Analysis of the 16S rRNA gene library

A total of 150 clones from each library (UH and GF) were sequenced. Prior to analysis, all chromatograms were trimmed with Phred-Phrap (http://www.phrap.com/phred/). Thirty-two chimeric sequences were identified and removed using Bellerophon v3.0 software (http://greengenes.lbl.gov). The clone sequences were clustered as Operational Taxonomic Units (OTU) using DOTUR (Schloss and Handelsman, 2005) and a cut-off of 97% of identity and further examined using rarefaction analysis. The richness and diversity indices were calculated using DOTUR, based on the non-parametric richness (ACE and CHAO1) and diversity (Shannon-Weaver and Simpson) indices with a cut-off of 100% (unique), 99% (0.01), 97% (0.03), 95% (0.05) and 90% (0.1) identity. Phylogenetic classifications were performed using RDPQuery (http://simo.marsci.uga.edu/public_db/rdp_query.htm) using the database parameters. The sequences were aligned using ClustalW (Thompson et al., 1994), and the distance was calculated using the Kimura 2 method (Kimura, 1980) using the DNADIST program (Kumar et al., 2004). The branches were tested with bootstrap analyses (1000 replications), and the layout of trees was designed using the online application “Interactive Tree Of Life” (iTOL) (http://itol.embl.de/) (Letunic and Bork, 2007).

3. Results and discussion

The focus of the present study was the dominant bacterial groups in the traps of the *U. hydrocarpa* (UH) and *G. filiformis* (GF) plants, which could contribute to plant nutrient acquisition. As the surface disinfection process could trigger the urchiles and eliminate the bacterial community inside the traps, we only washed the traps with sterilised distilled water. However, analysis based on Scanning Electron Microscopy showed that the contamination of bacteria from trap surfaces was insignificant. In addition, analysis based on the DGGE technique indicated that traps collected from different sites, for the same plant species, presented similar DGGE profiles (data not shown).

Although, many microorganisms were detected inside the *Utricularia* and *Genlisea* traps, the bacterial species were not identified. Therefore, after assessing the sequences for quality and the presence of chimaeras, a total of 268 sequences (141 from GH and 127 from UH) were used to identify the bacterial components in the traps. Most of the sequences clustered into *Firmicutes* (46.8%) and *Proteobacteria* (33.9%), indicating that these are the dominant bacterial phyla (Fig. 1 and S1) inside the traps. Minor bacterial phyla included *Acidobacteria* (9.3%), *Actinobacteria* (4.4%), *Bacteroidetes* (0.8%), *Chloroflexi* (0.4%), *Gemmatimonadetes* (0.4%), *Cytophagia* (0.4%), *Chlamydiae* (0.4%) and *Tenericutes* (0.4%) (Figs. 1 and S1). Krieger and Kourtev (2012a, 2012b) and Koopman and Carstens (2011) found similar phylum in the pitchers fluid of *Sarracenia purpurea* and *S. alata*, in which the *Proteobacteria*, *Bacteroidetes*, *Cytophagia* and *Firmicutes* were dominants. However, *Acidobacteria*, *Chlamydiae*, *Chloroflexi*, *Gemmatimonadetes* and *Tenericutes* were not detected in the evaluated pitchers (Table 1).
Fig. 1. Phylogenetic analysis of 248 bacterial 16S rRNA genes clones obtained from *G. filiformis* and *U. hydrocarpa*. The solid grey circles next to the tree branches correspond to bootstrap values higher than 70%. The tree display has one representative clone per OTU. A single OTU embraces all of the clone sequences sharing at least 99% similarity. The groups Tenericutes, Cyanobacteria, Actinobacteria, Firmicutes, Chlamydiae, Bacteroidetes, Acidobacteria, Chloroflexi, Gemmatimonadetes and Proteobacteria are as explained by the different colours in the legend. There were a total of 420 aligned nucleotide positions in the final data set.

Although some overlap of microbial populations between the two carnivorous plants (GF and UH) was observed, the communities were mostly distinct. Anaerobic *Clostridium* sp. and the facultative anaerobic, pectolytic *Dickeya* sp. (sin.: Pectobacterium sp.) were the dominant species in GH libraries, while the UH libraries were dominated by *Clostridium* sp., the facultative anaerobic *Aeromonas* sp. and the acidophilic, chemoorganotrophic and also facultative anaerobic *Acidobacterium* (Fig. 1). The characteristics of this bacterial community, mostly anaerobic or facultatively anaerobic, are in agreement with Adamec (2007), which observed that the environment inside the *Utricularia* trap is completely sealed and anoxic with limited periods of higher O₂ concentration after firing events. In addition, some genus, specifically *Acidobacterium*, thrive in low-pH environments and are capable of using complex organic substrates such as hemicellulose, cellulose and chitin (Ward et al., 2009), which could be obtained by digestion of algae and microcrustaceans, creating a microbial food web inside the traps, as previously suggested by Sirová et al. (2003). In *Utricularia*, these results therefore point to a microbial community well adapted to trap conditions with diurnally fluctuating oxygen conditions, lower pH, and copious supply of both complex and simple substrates. Much less is known about the physico-chemical conditions within *Genlisea* traps, but the lower diversity of microorganisms may be related to different trap structure and mechanism of trap functioning – these traps are opened and therefore there is less potential for a stable environment maintained by the plant.
The minor genera found in UH were Spiroplasma, Solirubrobacter, Lamia, Mycobacterium, Kinesioria, Microbacterium, Anaeromusa, Exiguobacterium, Flectobacillus, Arcicella, Terriglobus, Gemmatimonas, Cladulina, Plesiomonas, Dickeya, Citrobacter, Cronobacter, Methyloalkalum, Alkalibacterium, Duganella, Curvibacter, Pseudogulbenkiania, Bacteriovorax, Sphingomonas, Filomonocibium and Prosthecocibium, while in GF, the minor groups were Prochlorococcus, Actinoallomurum, Dendrosorobacter, Parachlamydia, Terriglobus, Aquitalea, Phenolbacterium and Magnetospirillum (Fig. 1), indicating that the microbial communities inside these UH and GF traps are different. In fact, based on the OTU classification on 97% similarity (OTU_{Almost}), only 1.2% of these species were found to be common to both UH and GF libraries.

Although more sequences from GF traps (141) than from UH traps (127) were evaluated, we found 62.5% more OTUs in GF than in UH, suggesting that the UH traps harbour a more diverse bacterial community as compared to GF traps. In fact, CHAO1 richness and Shannon–Weaver and Simpson diversity indices, determined at different dissimilarity levels (0.03, 0.05 and 0.10), revealed that the richness and diversity were significantly higher in UH traps than in GF traps. Additionally, the rarefaction curves suggested that the bacterial diversity at genus level was better sampled in GF than in UH (Fig. 52). As previously described (Adamec, 2007; Plachno et al., 2006, 2007), these carnivorous plant species live in different environments and employ different strategies to capture prey, which would explain this difference in bacterial diversity. In UH, the bacterial community present in the water surrounding the trap is sucked in together with the prey. If the conditions in the trap are favourable for some of the bacteria, then these species can establish a population during prey digestion. Accordingly, the diversity of the bacterial community is driven by a stochastic event (suction) and in the end by the physical–chemical conditions inside the trap. However, in GF, the microbial community inside the trap is carried mainly by the prey, although some motile bacteria can enter the GF trap using their flagella or other structures. However, to confirm this hypothesis, different Utricularia and Genlisea species should be evaluated, and the correlation between bacterial diversity and strategies for prey capture should be calculated.

The association between carnivorous plants and bacteria has been consistently described for Sarracenia spp. (Peterson et al., 2008; Siragusa et al., 2007; Krieger and Koutert, 2012a, 2012b; Koopman and Carstens, 2011; Koopman et al., 2010), but for the Utricularia and Genlisea genera the dominant bacterial species had not yet been identified. Therefore, in the present study, the dominant groups present in the bacterial community inside the traps of U. hydrocarpa and G. filiformis were described. Additionally, we suggest that this bacterial diversity could be driven not only by random assemblages of the bacterial community surrounding the plant, but also by the environment of the trap and the specific strategy used by the plant to capture its prey.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2013.12.008.

References


