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First Records of 'Flagship' Soil Ciliates in North America

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'Flagship' ciliates were investigated from soil samples collected in Florida, USA. This was undertaken to determine if species thought to be restricted to a given world region could be uncovered from similar habitats in a novel location, e.g. another continent. Two species of *Condylostomides* were discovered, and recorded from the North American continent for the first time. *Condylostomides etoschensis* was known only from Africa, but was found to be thriving in a Florida study site. An 18S rDNA sequence for this species was determined for the first time. Also discovered from the same study site was the ciliate *Condylostomides coeruleus*, previously known only from Central and South America. These two 'flagship' ciliates were found in the same habitat, from a continent well outside of their previously recorded biogeographies. Molecular sequencing and microscopy investigations were conducted to form the baseline for future work within this genus. Soil ciliates can obtain large population numbers and form cysts and are therefore likely able to disperse globally. These new records provide additional evidence that large distances, even between continents, do not hinder microbes from thriving globally. The absence of these conspicuously-colored gold and blue ciliates from previous studies is likely due to undersampling, rather than to any physical barriers.

Key words: Ciliates; soil; Florida; *Condylostomides etoschensis*; *Condylostomides coeruleus*; biogeography.

Introduction

Ciliated protozoa are extremely common in soil environments, despite frequently being in a cryptic state (Esteban et al. 2006). Ciliates in soil live within the micro water content surrounding soil particles (Finlay et al. 2001) and many are able to form cysts in order to survive adverse conditions (Bourland 2017). These cysts may remain viable for long periods of time (Foissner 2016), contributing to their dispersal (Finlay et al. 2001). Although a large ciliate population might not at first be readily detected in a given fresh soil sample, when environmental conditions change a dynamic community may develop as the ciliates excyst along with the growth of other protist and prokaryotic communities. Rewetting of soil samples stimulates ciliate excystment (Foissner et al. 2002) and reveals a community of ciliates, including cryptic species (Finlay and Fenchel 2001) that emerge as their preferred niche develops.

Ciliates in soil are integral members of the microbial loop (Azam et al. 1983) in both directions of trophic levels, acting not only as consumers but also as food for members of the soil community. As grazers on small protists and bacteria, ciliates are fundamentally important in healthy soils (Esteban et al. 2006; Foissner 2016). Ciliates feeding on bacteria within soils release nitrogen (NH_4^+) which is available as nutrients for plants (Ingham et al. 1985). Ciliates also feed on other protists, regulating these populations and providing additional micronutrients to the community. Ciliates are also important in the mineralization of nutrients in soil (Griffiths 1986) and are therefore beneficial to plant communities. The rates of carbon and nitrogen cycling in soil are stimulated by the ciliates present as grazers on bacterial communities (Finlay et al. 2000). It has been suggested that ciliates could be considered as bioindicators of soil health due to their responses to anthropogenic influences (Li et al. 2010).

Ciliates are well documented as inhabiting all states of soil oxygenation, from obligate anaerobes to aerophiles (Lynn 2008). This is in contrast to the pervasive beliefs of amateur gardeners found in various blogs and social media outlets such as on Instagram (Hines 2019b), that incorrectly assume the presence of ciliates in soils is indicative of exclusively anaerobic, and allegedly ‘unhealthy’ conditions despite inadequate literature to support this. The community of trophic soil ciliates present in a given area changes over time at small spatial scales and is influenced by factors such as daily fluctuations in water content (Finlay et al. 2000). As such, soil ciliate communities are capable of rapid change, with both total excystment and blooms possible.

Ciliates are common within all soils (Bamford 1995; Bates et al. 2013) and are important members of microbial communities in all global regions. Soil ciliates are thought to form cysts more readily in areas that experience dryness (Foissner et al. 2002) rather than rainforest habitats which maintain constant moisture (Foissner 1997). It is possible that more saturated soils act in a similar way to freshwater habitats, such that they should be examined immediately after sampling as their community is more active (i.e. not encysted), and vulnerable to change.

Although a wealth of ciliate diversity is reported to exist in soils, ciliate biodiversity in general in sparsely recorded (Venter et al. 2018). New species of soil ciliates are still being described from 'well-searched' areas such as Europe (Foissner et al. 2005), which confirms that the extent of soil ciliate biogeography and biodiversity is still undetermined. Examples of 'flagship' soil ciliates exist in the literature that are described as endemic to a particular region such as Africa (Foissner et al. 2002) or South America (Foissner 2016).

Borrowed from the field of wildlife conservation, the term "flagship" refers to ciliates whose morphological distinctiveness is such that their presence should not be missed in any environmental sample. The term as applied to ciliates is used in a unique way, distinct from that commonly used in megafaunal conservation; rather than a species selected to raise conservational awareness, flagship ciliates are used to investigate the potential for restricted biogeographic distributions and endemism (Foissner 2006). As such they have been considered the "ultimate proof" for testing biogeographical theories surrounding microbial endemism (Foissner 2006; Foissner et al. 2008; Segovia et al. 2017) and can be a useful tool for better understanding taxa with unknown spatial distributions (Andelman and Fagan 2000). The idea that flagship ciliates are 'proof' for microbial endemism is perhaps a flawed concept: when the size of the globe is considered with the astronomical number of niches compared with the number of microbial ecology researchers present in any given area, it is likely that large parts of the planet remain unexplored for microbial diversity and, even for areas which have been studied, that effort may not be exhaustive.

Flagship ciliates represent an ideal target when seeking a better understanding of ciliate biogeography, including soil communities (Bourland 2017). Since Florida had never benefited from an investigation of its soil ciliates, it represents a significant knowledge gap for this group of organisms. A single report of a sample collected from Everglades National Park revealed a new species (Foissner 2016), but it is unclear whether this species is limnetic due to the swamp habitat

in which it was collected. Florida has not benefited from additional soil ciliate diversity campaigns, despite its interesting geographic location within the subtropics.

Due to the vast literature on soil ciliates from global regions (Foissner et al. 2016 and references therein), Florida soil samples were occasionally taken in conjunction with sampling of freshwater habitats during ciliate biodiversity and biogeography surveys within Florida (Hines 2019a). When freshwater sites dried up during drought conditions, some sediment from these once aquatic habitats was collected and rewetted. None of the targeted freshwater species were recovered using this technique, however, a different (i.e. soil adapted) community was observed. It should be noted that no soil 'flagship' ciliates were recovered from any limnetic sampling during the course of the survey.

As a result of this limited study of Florida soils, one site was found to be very productive: an abandoned natural wooded area on the Harbor Branch Oceanographic Institute campus. This site yielded two 'flagship' ciliate species: one is the first record of the species outside of Africa, and the other is the first record for North America.

Results

The average water content of soils collected at the sampling site was 18.47%. The remaining solid fraction had an average Total Organic Matter of 8.06%. The average grain size breakdown was: 0.62% gravel, 96.95% sand, and 2.42% fines. At a temperature of 23°C the pH was 7.60 and the salinity was 0.06 (PSU), i.e. equivalent to fresh water.

Laboratory cultures from freshly collected soils contained diverse ciliate populations including two ciliates which stood out due to their size and color. Based on their morphology these cells were identified as *C. etoschensis* and *C. coeruleus*. These laboratory cultures commonly gave densities for *Condylostomides etoschensis* of 5 cells mL^{-1} and were stable for at least six months when maintained with water and food at 30 °C. Soil cultures which were over saturated (nearly flooded) and overfed (triple amount of farro wheat grains) and then incubated at 30℃ showed the best results for growth of 'flagship' ciliate targets and overall ciliate biomass (e.g. small Hypotrichs and *Colpodea*) with densities of *C. etoschensis* reaching 35 cells per mL within the

147 first week.
148
149 ***Condylostomides etoschensis*** **Foissner, Agatha and Berger, 2002**
150 *C*. *etoschensis* is distinct due to its bright gold coloration and large oral aperture (Fig. 1) which
151 distinguish it from other common soil species. The cells found in Florida samples matched the
152 description of *C*. *etoschensis* by Foissner et al. (2002).

153 A large contractile vacuole in the cell's posterior end was described for the African cells
154 (Foissner et al. 2002), which deforms the cell when full. This was also observed in Florida cells,
155 along with the adoral zone of membranelles (AZM) being long and conspicuous. The oral aperture
156 was wide and occupied nearly 50% of cell length.

157 The type location, and only site of observation in Africa, was within a "highly saline soil"
158 (although no data were given) from an ephemeral pool in Etosha Pan, Namibia (Foissner et al.
159 2002). Conjugation was recorded in the African strains in which two cells lock onto each other at
160 the oral aperture and exchange genetic material. Although rarely observed, this was also recorded
161 in Florida samples (Figure 1B). Cells were observed to stay in this state for over 1 hour.

162 Cysts were observed and well documented from the African site. Cysts with a similar
163 appearance were recorded in Florida, however, these were never directly observed to excyst.
164 Based on soil habitat, overall morphology, size, and unusual gold coloration from cortical
165 granules (Table 1) the species was confirmed to be C. *etoschensis*. No molecular sequence was
166 provided in the diagnostic literature (Foissner et al. 2002) and the species had not been recorded
167 since, including from similar sampling campaigns in South America (Foissner 2016 and references
168 therein).
169 Finding this species in North America is the first record outside of its original African
170 range, at a distance of ~12,000 km from its documented discovery habitat, and suggests that this
171 and other soil ciliate species can overcome barriers to dispersal such as distance.
172
173 ***Condylostomides coeruleus*** **Foissner, 2016**
174 On average, only two *C*. *coeruleus* cells per mL could be found in productive samples after
175 thorough searching.

During investigations of *C. etoschensis* in Florida (see above), this morphologically-similar but blue-colored species was found within the same subsamples coming from the same cultures. Based on habitat type, morphology and coloration this species was identified as *C. coeruleus* (Fig. 2). Detailed morphometrics (Table 1) were obtained to compare the Florida species to the diagnostic literature (Foissner 2016).

Molecular Phylogeny

We sequenced the 18S rRNA gene from both *C. etoschensis* and *C. coeruleus*. The *C. etoschensis* amplicon was approximately 1510bp: FL1, MK543444 (1,505bp), FL2 MK543442 (1,513bp), and FL3, MK543443 (1,501bp). The three sequences clustered closely (Fig. 3) and were clustered with, but distinct from, the other *Condylostomides* species in GenBank. The DNA from *C. coeruleus* amplified poorly and we were only able to sequence the gene in one direction with an amplicon size of 799bp (FL1, MK543445). Despite this, the isolate clusters with the only sequence available in GenBank for *C. coeruleus* (Fig. 3; [*C. coeruleus* SLS-2007 AM713188, 98% (784/799) base pairs matched], Schmidt et al. 2007; Foissner, 2016). Our isolate also clusters with a second sequence from a *Condylostomides* not identified to species level (Fig. 3; KP970236, 98% (785/799) base pairs matched).

Many heterotrichs have been sequenced, with several examples of *Condylostomides* currently available in Genbank. However, since molecular data for *C. etoschensis* did not exist in the literature, the Florida record is the baseline for future work within this genus and for other global biodiversity studies that may encounter this cell. The Florida cell is related only sequences available for *Condylostomides*, and also clusters with *Linostomella* sp. as predicted in the diagnostic literature (Lynn 2008) (Fig. 3). Although, at the morphological level, the gold and blue *Condylostomides*, respectively, appear closely related, at the molecular level they are related but distinct.

Observations on Laboratory Cultures

To test the response of cultures to adverse conditions, triplicate soil cultures were prepared, examined and found to contain the target flagship soil ciliates. These cultures were left to incubate at 30℃ for 3 months. Without water being added, the cultures were completely dry in less than a week. After 3 months the cultures were restarted and treated as described to stimulate excystment.

A stable and similar ciliate population developed. This included the population of target flagships at the same densities as previously recorded. A previously productive soil sample 'forgotten' in the 30°C incubator was rewetted after being untouched for more than one year, and a similar microbial consortium appeared, including similar densities of the target C. *etoschensis* despite total desiccation during this time. Similarly, a soil sample frozen at -20 °C immediately after collection and stored, frozen, for 1 year was restarted as previously described. A similar, but less active, microbial consortium developed and the target ciliate *C. etoschensis* was recorded from this sample.

Discussion

The 'Flagship' soil ciliates investigated here were all isolated from rewetted soil samples and were never found in freshwater samples. The genus *Condylostomides* has been reported from a wide variety of habitats and geographies, such as the freshwater *C. groliere* from Europe (Silva Neto 1994), and species such as *C. vorticella* from brackish waters of Africa (Dragesco and Dragesco-Kernéis 1986) and *C. nigra* from Europe (Lake Geneva), which has a distinct similarity to *C. coeruleus* including size and color (Dragesco 1960). The new record of these soil flagship representatives in North America further expands the global biogeography for this group. The target ciliate cysts for the species described here were apparently always present in soil samples from the discovery site over the course of sampling for over one year, as the species were always found after rewetting the soil samples. Florida site over the course of sampling for over one year. Gold colored cysts, likely belonging to *C. etoschensis*, were found in the soil samples, sometimes in numbers > 20 mL^{-1}. Despite numerous attempts these were never directly observed to excyst. The description of the African cysts (Foissner et al. 2002) matches that of the cysts observed in Florida samples. This ciliate was previously described only from Namibia, Africa (Foissner et al. 2002), despite numerous soil investigations from other global habitats (Foissner et al. 2008; Foissner 2016 and references therein) leading to the claim that this species was endemic to that world region.

Fresh dry soil may have few active ciliates present, but a vast number may be recorded later as the amount of water increases, due to excystment of ciliates. The large number of cysts present in soils (Foissner et al 2002) ensures the survival of a stable ciliate population under all environmental conditions, and consequently all natural soils contain ciliates. The target flagship soil ciliates were shown to be resistant to unfavorable environmental conditions, with cysts still viable from samples after one year of dry incubation at 30 °C or freezing at -20 °C.

The soil communities of Florida were found to contain relatively few species when examined directly from the field, and even after 24 hours only small Colpodea were observed. After two days a more diverse community developed following excystment. At a global level, ciliate soil diversity is unresolved due to undersampling (Chao et al. 2006) which confounds ciliate diversity and biogeographies at all levels. It is likely the natural bacterial and small protist community takes time to develop under incubation, and it is only when these levels have increased that ciliate excystment occurs in high enough numbers to be detected (Foissner et al. 2002).

These conditions proved most productive for smaller protists and bacteria to flourish and these serve as the food sources for target ciliates. The literature suggests that the oversaturation of cultures or allowing them to 'spoil' negates ciliate species development (Foissner et al. 2002) which is a rule likely true for most samples. The Florida cultures, however, required larger amounts of water and higher food availability to reveal the flagships in greatest density. Standard methods (Foissner 2016) were followed with success, but the two flagship targets were most prevalent when cultures were treated as described above.

As reported in the literature, investigations of terrestrial ciliates from South America (Foissner 2016) revealed new species, including the conspicuous species *Condylostomides coeruleus*. This species was not recorded from similar soil campaigns in African habitats (Foissner et al. 2002). Due to this apparently restricted biogeography this blue ciliate was recently described as a 'flagship' with a biogeography limited to the previous discovery sites explored in South and Central America (Foissner 2016).

This species has been described as an "endemic Gondwana flagship" (Foissner 2016), this was despite being reported in the same text as Central American areas which were not part of a Gondwana breakup. The new record from Florida, a geologically recently emerged habitat (Watts 1969), disprove the alleged restriction. It is surprising though that the gold *Condylostomides*

etoschensis was never recorded in South American investigations, but is likely a result of undersampling of ciliates and known difficulty with detection of species even if present.

C. coeruleus was always found in subsamples that also contained *C. etoschensis*. Although appearing blue in color under high-power magnification, when using a dissecting microscope (used for picking of cells and initial observations) they appeared nearly colorless, such that their overall movement type rather than color was used as the indicator for picking cells. No other species of soil *Condylostomides* were observed during these investigations. The Florida observations of *C. coeruleus* was smaller than that reported in the literature. Florida measurements were made on cells taken from fresh cultures, and this may not have allowed the species to grow to its full size. All other morphological diagnostics match those described in the literature (Foissner 2016).

Molecular comparisons are now possible to further investigate this genus. The ciliate *Linostomella* sp. was theorized as being the closest relative to *Condylostomides* (Foissner et al. 2002; Lynn 2008). The new sequences and phylogenetic tree for *C. etoschensis* reported here supports this relationship.

It is clear from these results that C. *coeruleus and C. etoschensis* can thrive within the same ecological habitat. The habitat they require, and the environmental factors that stimulate excystment are evidently present in the Florida soils investigated. The two species were always found together during this project. No cysts were directly observed that match the Venezuelan description of *C. coeruleus*: bluish and about 100µm in diameter (Foissner 2016). It is possible that even if present in high numbers they were obscured by the soil particles they may attach to, and were therefore overlooked during this investigation. The original description suggests the possibility for this species to be 'common in slightly to moderately saline habitats' (although no data values were given) of South and Central America (Foissner 2016). The species was thought to be a litter or limnetic species based on its blunt shape (Foissner 2016); however, the Florida soil habitat was found to be mostly sandy with organic material. This species was never recorded in limnetic samples investigated during this project.

Conclusions

The diversity of ciliates in any habitat is still poorly investigated, with both new species awaiting discovery, and 'flagship' ciliates being recorded from new biogeographies. The discovery of two flagship soil ciliates in Florida, with minimal sampling effort revealed the first record outside of

297 Africa for *Condylostomides etoschensis*, which is further evidence for the ability of ciliates to
298 disperse globally. The first record for North America of *Condylostomides coeruleus* is additional
299 evidence that species thought to be restricted to South and Central America can overcome this
300 geographic barrier and thrive within Florida, and likely other habitats at a global level. Sequences
301 for flagship ciliates alleged to have restricted biogeography (Foissner et al. 2008) simply do not
302 exist in databases (Schmidt et al. 2007), with only a handful present at the time of writing.
303 Deposition of the three *C. etoschensis* sequences will allow for future researchers to compare their
304 study sites to the Florida baseline. The ability of soil ciliates to readily form cysts, as well as exhibit
305 conspicuous coloration makes them good candidates to test for ciliate biogeography. As sampling
306 efforts increase, these and other soil ciliates will probably have their biogeographic distributions
307 expanded.
308 Florida has been shown to harbor freshwater flagship species originally proposed to be
309 restricted to a given biogeography (Hines 2019a; Hines et al. 2016), and species once thought to
310 be restricted often are found in further regions as sampling efforts increase (Hines et al. 2018;
311 Esteban et al. 2001; Finlay 2002). Soil samples were taken sporadically in addition to intensive
312 sampling of freshwater habitats. As such, these results although novel, are by no means exhaustive,
313 and likely many other flagship soil taxa await discovery in Florida. This investigation of soils
314 suggests that Florida is both capable of harboring a diverse ciliate community, and that soil
315 'flagships', like freshwater 'flagships', can spread to global regions wherever they find their
316 preferred ecological niche.
317
318
319 ## Methods
320
321 **Study site:** The sampling location site surrounds a wild growing *Citrus* tree resembling in
322 appearance and taste *Citrus aurantium* (known commonly as "bitter orange" or "Seville orange")
323 located within an old, unmanaged, wooded area with the fruit falling and rotting back into the
324 ground. Numerous smaller orange trees were found to be germinating within several meters. The
325 tree is within a densely wooded area and the site has been untouched for at least 50 years. The site
326 is rich with insects of the family Culicidae (Mosquitos) confirming that it is chemically untreated.
327 The site is located at 27°31'53.1"N 80°21'18.3"W in St. Lucie County, Florida.

Soil samples: The soil is largely sandy (white 'sugar sand') with dense organic material mixed throughout, and some leaf litter present. Top soil layers down to 1.5 cm were collected using a sterile metal scoop and transferred into sterile 125 mL Nalgene bottles.

Soil pH and salinity: Standard methods were followed to obtain soil data (Finlay et al. 2000). Samples were freshly collected and dried overnight at 60˚C. This material was then sieved (2mm) to remove large organics. A 1:5 soil/water suspension was made with 60g soil to 300mL deionized water (DI H_2O) and stirred for 30 minutes. The sample was allowed to settle for 15 minutes. The pH and salinity of the solution were determined using a YSI probe (four port "Digital Professional Series", Xylem, Yellow Springs OH, USA).

Soil type: Soil samples were collected in triplicate and processed for soil characteristics within an hour of collection using the following techniques (modified from Folk 1974; Dean 1974).

Approximately 60g of soil from each replicate was dried for 1 hour at 60°C and clumps were broken apart using a mortar and pestle. Samples were then sieved through 2 mm and 0.063 mm sieves to separate the gravel (> 2mm), sand (2mm to 0.063 mm), and fines (< 0.063 mm) into fractions (Folk 1966). The sieves were shaken by hand for ten minutes and each of the fractions was rinsed into separate pre-weighed beakers using deionized water. Samples were dried in a 60°C oven for 48 hours. Each fraction's absolute weight was divided by the total of all three fractions to calculate the percentage.

Water content was determined by drying ~30g of soil in glass Petri dishes for 48 hours at 60 °C. The dried sediment was ground briefly using a mortar and pestle and sieved to remove the fraction above 2mm which was used for total organic matter analysis: One gram of the fraction was put into ceramic crucibles and heated for 4 hours in a 550 °C pre-heated muffle furnace. The organic content was determined from weight loss and reported as % Total Organic Matter.

Soil cultures: Soil cultures were started within 1 hour of collection by placing ~50g soil in sterile 9 cm glass Petri dishes (Pyrex) and wetting with ~25 mL sterile deionized H_2O. The dish was swirled to mix in the overlaying floating soil particles. Grains of farro wheat (*Triticum* sp.) were prepared by boiling in deionized H_2O for ~15 minutes and then allowing them to cool for 10 minutes in fresh sterile deionized H_2O. Grains were squashed by hand and were added to the cultures with one at the edge and one in the center of the Petri dish, such that each grain was half submerged and half above water/sediment line to encourage fungal growth. The lid was placed on the Petri dishes and cultures were incubated at 25 °C, 30 °C, and 37 °C. After 24 hours of

incubation the enriched cultures were examined every 24 to 72 hours for periods up to several weeks. Water was added as needed as incubation caused drying. New farro grains were added when breakdown (e.g. consumed by bacteria, fungi and worms) had occurred.

In order to sample the enriched cultures, they were held at a slight tilt and a sterile pipette was used to transfer the top runoff water at an edge onto an observation chamber. Due to the relatively low amount of water in these concentrated soil cultures, after observation this liquid was returned to the culture, with additional deionized H_2O added as needed.

Microscopy: Individual ciliate cells were picked using a micropipette under a dissecting microscope for DNA extraction, culture, or onto welled slides for further examination under higher powered microscopy. Initial observations were made using a 1 mL Sedgewick-Rafter counting chamber which allowed observation, enumeration and photography.

A fully equipped Olympus BX-53 microscope was used for detailed observation and photomicroscopy. An Olympus DP72 camera and its associated software (cellSens v1.17) was used to record images.

DNA extraction: REDExtract-NAmp PCR ReadyMix (Sigma Aldrich) was used for both extraction and amplification of the single cell samples. The method followed the 'saliva' protocol described by Kim and Min (2009). Samples were either amplified immediately or stored at -20 °C. Amplification used the Euk-82F and EukB primers (Elwood et al. 1985; Medlin et al. 1988; Integrated DNA Technologies (Coralwood, IA, USA)). Sequences obtained from these single cell samples were deposited into GenBank.

Sanger sequencing was conducted by MCLab (South San Francisco, CA, USA). Analysis was performed using the software packages within the DNAStar Lasergene 12 Core Suite which allowed editing of sequences and the creation of contigs. Sequences were aligned using MEGA version 10.0.5.

Phylogenetic analysis: The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-10674.67) was used. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then

selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2602 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018) and the phylogenetic tree was edited using Interactive Tree of Life (iTOL) version 5 (Letunic and Bork 2019).

'Declarations of interest: none'.

References

Andelman S, Fagan W (2000) Umbrellas and flagships: efficient conservation surrogates or expensive mistakes? Proc Natl Acad Sci USA **97**:5954-5959

Azam F, Fenchel T, Field JG, Grey JS, Meyer-Reil LA, Thingstad F (1983) The ecological role of water-column microbes. Mar Ecol Progr Ser **10**:257-263

Bates S, Clemente J, Flores G, Walters W, Parfrey L, Knight R, Fierer N (2013) Global biogeography of highly diverse protistan communities in soil. ISME J **7**:652

Bamforth S (1995) Interpreting soil ciliate biodiversity. Plant Soil **170**:159-164

Beers CD (1948) Excystment in the ciliate *Bursaria truncatella*. Biol Bull **94**:86-98

Bourland W (2017) How far do ciliate flagships sail? A proposed Gondawanaland endemic species at anchor in Idaho soils. Protist **168**:352-361

Chao A, Li P, Agatha S, Foissner W (2006) A statistical approach to estimate soil ciliate diversity and distribution based on data from 5 continents. Oikos **114**:479-493

Dean W (1974) Determination of carbonate and organic matter in calcareous sediments and sedimentary rocks by loss on ignition: comparison with other methods. J Sediment Petrol **44**:242-248

Dragesco J (1960) Ciliés mésopsammiques littoraux. Systématique, morphologie, écologie. — Trav Stn biol Roscoff **122**:1–356

Dragesco J, Dragesco-Kernéis A (1986) Ciliés libres de l'Afrique intertropicale. Introduction à la connaissance et à l'étude des Ciliés. Faune tropicale (Éditions de l'Orstom, Paris) **26**:1-559.

Elwood H, Olsen G, Sogin M (1985) The small-subunit ribosomal RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. Mol Biol Evol **2**:399-410

Esteban GF, Finlay BJ, Charubhun N, Charubhun B (2001) On the geographic distribution of *Loxodes rex* (Protozoa, Ciliophora) and other alleged endemic species of ciliates. J Zool **255**:139-143

Esteban GF, Clarke KJ, Olmo JL, Finlay BJ (2006) Soil protozoa—an intensive study of population dynamics and community structure in an upland grassland. Appl Soil Ecol **33**:137-151

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39**:783-791

Foissner W (1997) Soil ciliates (Protozoa: Ciliophora) from evergreen rain forests of Australia, South America and Costa Rica: diversity and description of new species. Biol Fertility Soils **25**:317-339

Foissner W (2006) Biogeography and dispersal of micro-organisms: a review emphasizing protists. Acta Protozool **45**:111-136

441 **Foissner W** (2016) Terrestrial and semiterrestrial ciliates (Protozoa, Ciliophora) from Venezuela
442 and Galápagos. Denisia **35**:1-912

443 **Foissner W, Agatha S, Berger H** (2002) Soil ciliates (Protozoa, Ciliophora) from Namibia
444 (Southwest Africa), with emphasis on two contrasting environments, the Etosha region and the
445 Namib Desert. Denisia **5**:1-1459

446 **Foissner W, Chao A, Katz L** (2008) Diversity and geographic distributions of ciliates (Protista:
447 Ciliophora). Biodivers Conserv **17**:345-363

448 **Foissner W, Berger H Xu, K Zechmeister-Boltenstern S** (2005) A huge, undescribed soil ciliate
449 (Protozoa: Ciliophora) diversity in natural forest stands of Central Europe. Biodivers Conserv
450 **14**:617-701

451 **Folk R** (1966) A review of grain-size parameters. Sedimentology 6:73-93

452 **Folk R** (1974) Petrology of Sedimentary Rocks. Hemphill Publishing Co, Austin, Texas, 170 p

453 **Finlay B** (2002) Global dispersal of free-living microbial eukaryote species. Science **296**:1061-1063

454 Finlay B, Fenchel T (2001) Protozoan community structure in a fractal soil environment. Protist
455 **152**:203-218

456 **Finlay B, Esteban G, Clarke K, Olmo J** (2001) Biodiversity of terrestrial protozoa appears
457 homogeneous across local and global spatial scales. Protist **152**:355-366

458 **Finlay B, Black H, Brown S, Clarke K, Esteban G, Hindle R, Olmo J, Rollett A, Vickerman K** (2000)
459 Estimating the growth potential of the soil protozoan community. Protist **151**:69-80

460 **Griffiths B** (1986) Mineralization of nitrogen and phosphorus by mixed cultures of the ciliate
461 protozoan *Colpoda steinii*, the nematode *Rhabditis* sp. and the bacterium *Pseudomonas*
462 *fluorescens*. Soil Biol Biochem **18**:637-641

Hines H (2019a) The biogeography, phylogeny, and dispersal of freshwater and terrestrial free-living ciliates in Florida, USA (Doctoral dissertation, Bournemouth University)

Hines HN (2019b) Cell-fies: sharing microbiology with global audiences through Instagram.FEMS Microbiol Lett **366**:fnz205

Hines H, McCarthy P, Esteban G (2016) The first record for the Americas of *Loxodes rex*, a flagship ciliate with an alleged restricted biogeography. Microb Ecol **71**:5-8

Hines H, Onsbring H, Ettema T, Esteban G (2018) Molecular investigation of the ciliate *Spirostomum semivirescens*, with first transcriptome and new geographical records. Protist **169**:875-886

Ingham R, Trofymow J, Ingham E, Coleman DC (1985) Interactions of bacteria, fungi, and their nematode grazers: effects on nutrient cycling and plant growth. Ecol Monogr **55**:119-140

Kim S, Min G (2009) Optimization of DNA extraction from a single living ciliate for stable and repetitive PCR amplification. Animal Cells Systems **13**:351-356

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol **35**:1547-1549

Letunic I, Bork P (2019) Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res **47**:W256-W259

Li J, Li M, Yang J, Ai Y, Xu R (2010) Community characteristics of soil ciliates at Baiyun Mountain, Guangzhou, China. Zool Stud **49**:713-723

Lynn D (2008) The Ciliated Protozoa: Characterization, Classification, and Guide to the Literature. 3rd edn, Springer Science & Business Media, New York, 605 p

Medlin L. Elwood H, Stickel S, Sogin M (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71**:491-499

Segovia B, Dias J, Cabral A, Meira B, Lansac-Tôha, F., Lansac-Tôha F, Bini L, Velho L (2017) Common and rare taxa of planktonic ciliates: influence of flood events and biogeographic patterns in neotropical floodplains. Microb Ecol **74**:522-533

Schmidt S, Foissner W, Schlegel M, Bernhard D (2007) Molecular phylogeny of the Heterotrichea (Ciliophora, Postciliodesmatophora) based on small subunit rRNA gene sequences. J Eukaryot Microbiol **54**:358-363

Silva Neto ID (1994) Morphologie et ultrastructure du cilié *Condylostomides grolieri* gen. n. sp. n. [Ciliophora: Heterotrichida]. Acta Protozool **33**:149-158

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol **10**:512-526

Venter P, Nitsche F, Scherwass A, Arndt H (2018) Discrepancies between molecular and morphological databases of soil ciliates studied for temperate grasslands of central europe. Protist **169**:521-538

Figure 1

Click here to access/download;Figure;Gold ciliate figure 600 tiff1.tiff

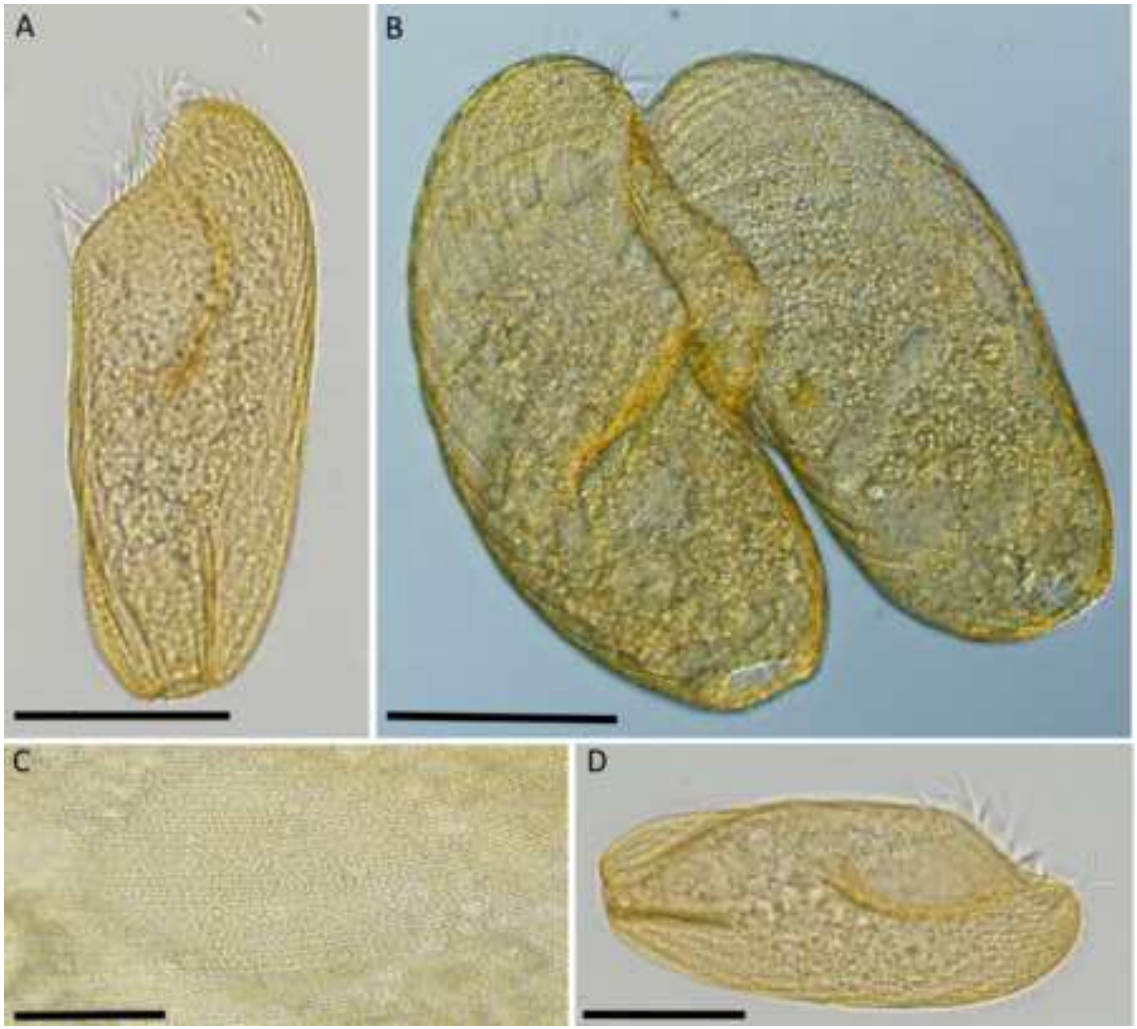
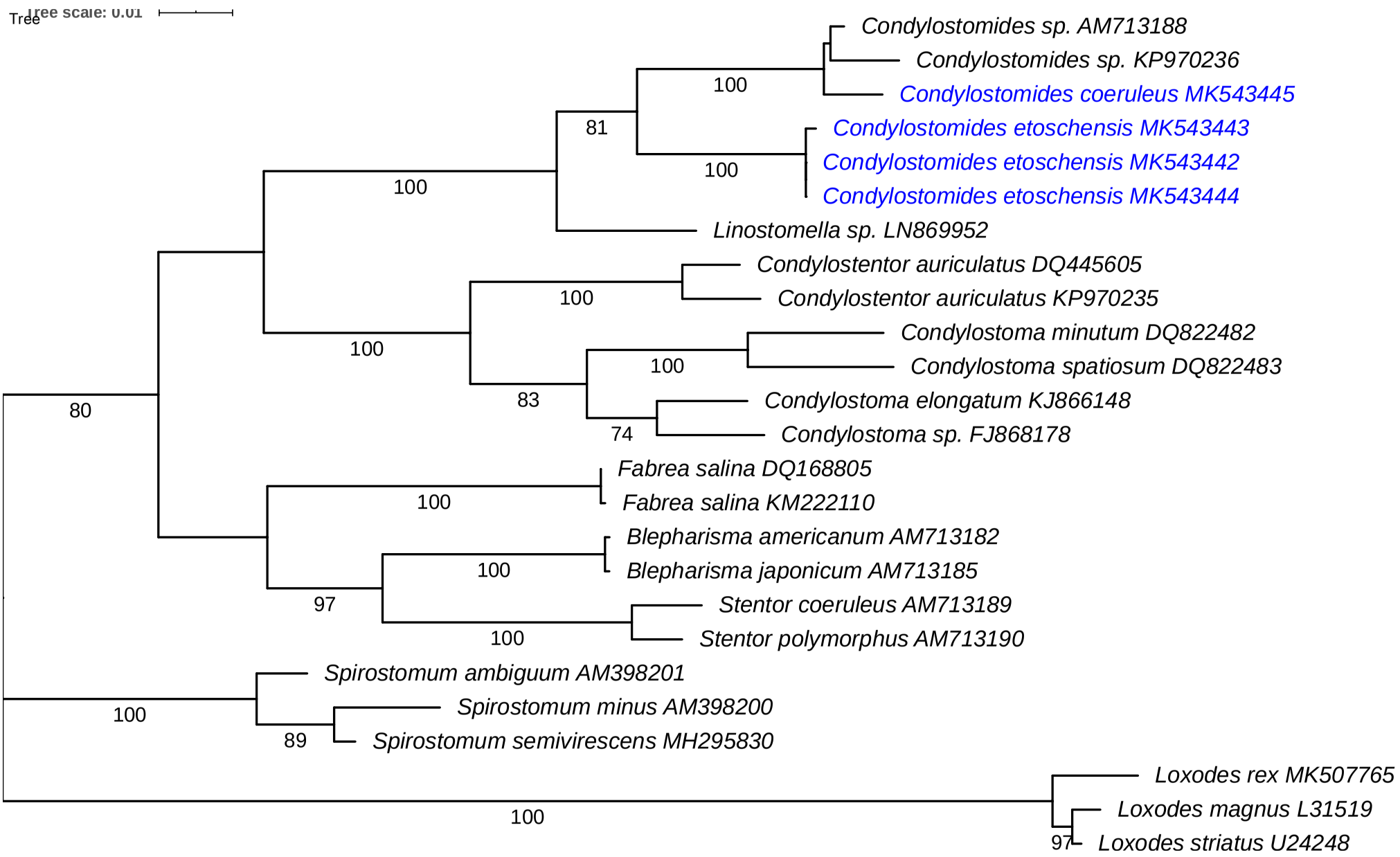


Figure 2

Click here to access/download;Figure;Blue soil ciliate figure 600 tiff.tiff


Tree scale: 0.01
Condylostomides sp. AM713188
Condylostomides sp. KP970236
Condylostomides coeruleus MK543445
Condylostomides etoschensis MK543443
Condylostomides etoschensis MK543442
Condylostomides etoschensis MK543444
Linostomella sp. LN869952
Condylostentor auriculatus DQ445605
Condylostentor auriculatus KP970235
Condylostoma minutum DQ822482
Condylostoma spatiosum DQ822483
Condylostoma elongatum KJ866148
Condylostoma sp. FJ868178
Fabrea salina DQ168805
Fabrea salina KM222110
Blepharisma americanum AM713182
Blepharisma japonicum AM713185
Stentor coeruleus AM713189
Stentor polymorphus AM713190
Spirostomum ambiguum AM398201
Spirostomum minus AM398200
Spirostomum semivirescens MH295830
Loxodes rex MK507765
Loxodes magnus L31519
Loxodes striatus U24248
100
81
100
100
100
100
100
83
74
80
100
100
97
100
100
89
100
97
100

	Condylostomides etoschensis		*Condylostomides coeruleus*	
Location	Africa	Florida	South America	Florida
Cell length (µm)	160- 300 (mean 240)	165-310 (mean 225)	150-315 (mean 235)	110-220 (mean 160)
Cell width (µm)	70- 150 (mean 110)	70-150 (mean 110)	85-155 (mean 120)	40-64 (mean 55)
Moniliform macronucleus	1	1	1	1
Number of micronuclei	~21	ND	ND	ND
Macronucleus size	⅔ cell length	⅔ cell length	⅔ cell length	⅔ cell length
Nodule number	~8	~8	~9	~9
Nodule length (µm)	~25	~25	~25	~25
Contractile vacuole	Present	Present	Present	Present
Kineties	37	~40	39	~40
Color	Gold	Gold	Blue	Blue
Molecular sequence	No	Yes	Yes	Yes

Table 1. Morphometrics for *Condylostomides etoschensis* discovered in Florida compared to the original description recorded in Africa (Foissner et al. 2002) and for *Condylostomides coeruleus* discovered in Florida compared to the original description from South America (Foissner 2016). The Florida cell matches to that described from the literature (Schmidt et al. 2007; Foissner 2016).

Figure 1. Flagship soil ciliate *Condylostomides etoschensis* from Florida (USA).

A: *in vivo* image. The ciliate is swimming and the natural gold color is clear in brightfield microscopy. Scale bar 100 µm.

B: the two cells are joined in conjugation at the mouth to exchange genetic material. Scale bar 100 µm.

C: a close up of the cell's cytoplasm showing the ciliary rows and cortical granules which cause the gold coloration. Scale bar 10 µm.

D: the large oral aperture at upper right is conspicuous in this in vivo image, as well as the long Adoral Zone of Membranelles. Scale bar 100 µm.

Figure 2. *Condylostomides coeruleus in vivo* from Florida (USA).

A: brightfield microscopy showing distinct blue green coloration of a swimming cell. Oral aperture at upper left. Scale bar 40 µm.

B: the cell is feeding off bacteria surrounding soil particles. Scale bar 40 µm.

C: view of oral aperture (top) and ciliary rows leading down to terminal vacuole of *C. coeruleus*. Long visible above oral aperture. The blue hue of the cell's coloration is obvious under DIC microscopy. Scale bar 40 µm.

Figure 3. Phylogenetic tree of the Heterotrichea inferred from nuclear small subunit (SSU) r DNA sequences using the Maximum Likelihood method and Tamura-Nei model. The karyorelctean species *Loxodes striatus*, *Loxodes magnus*, and *Loxodes rex* were chosen as the outgroup. The *Condylostomides coeruleus* and *Condylostomides etoschesis* sequences generated during this project are indicated in blue. The phylogeny of these species is: Eukaryota; Alveolata; Ciliophora; Postciliodesmatophora; Heterotrichea; Heterotrichida; Condylostomatidae; *Condylostomides*.

Author conflict of interest:
NONE