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Identifying invaders: the case of *Ceratium furcoides* (Gonyaulacales, Dinophyceae) in South America

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Running head title: *Ceratium furcoides* in South America

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ABSTRACT

Ceratium furcoides is an invasive freshwater dinoflagellate that in the last three decades has expanded its geographic distribution in South America, being recently found in Paraná River floodplain (Argentina). Despite growing concern about the presence and impacts of this invader, information regarding genetic diversity in the southern hemisphere is missing. This work constitutes the first phylogenetic characterization of *Ceratium* populations of South America, particularly, from the Paraná system. After taxonomic identification as *C. furcoides* based on morphological traits, two sequencing-based approaches were applied using the ribosomal 18S gene: Sanger sequencing to isolated individuals, and high throughput amplicon sequencing (HTS) to environmental DNA. The sequence of *C. furcoides* obtained shared 100% identity to Asian sequences, and formed a highly supported clade in the constructed reference phylogenetic tree. HTS helps to recover low frequency genetic variants suggesting the presence of different population of *C. furcoides*, and to alert potential invasion in its early stages.

KEYWORDS: dinoflagellate; biological invasion; 18S rRNA gene; phylogeny.

List of abbreviations: **ASVs:** Amplicon Sequence Variants; **BLAST:** Basic Local Alignment Search Tool; **TE buffer:** Tris + EDTA buffer

The proliferation of non-native species is one of the greatest threats to ecosystems since it can alter the diversity of native species and ecological functioning. The dinoflagellate *Ceratium* is a conspicuous organism typically found in the northern hemisphere, Africa and Australia. It was first reported in South America in the 1990s and, as far as it is known, two species are facing invasive processes in this region: *C. furcoides* and *C. hirundinella* (Guerrero and Echenique 1997, Boltovskoy 2005). Particularly, *C. furcoides* was observed for the first time in Colombia (Ramírez-R et al. 2005) and started being widespread from Brazil to other regions (Santos-Wisniewski et al. 2007, Cavalcante et al. 2013). It was mainly found in reservoirs and eutrophic ecosystems (Oliveira et al. 2011, Almanza et al. 2016, Bordet et al. 2017). However, the first record in Argentina was in an alluvial lake of the middle Paraná River in 2009 (Barreda 2015), and then it was found in other stretches (Jati et al. 2014, Meichtry et al. 2016).

The identification of *Ceratium* species is based on morphological features that could be hampered by phenotypic plasticity and requires taxonomic expertise. Improved detection can be achieved by high-throughput sequencing techniques that could help for accurate differentiation of species and detection of low population abundances due to their high sensitivity.

Herein, we performed the first report of *Ceratium furcoides* in South America combining morphological, molecular and phylogenetic information of natural population from Paraná fluvial system. This system is the second largest river of South America, formed by a main channel and a floodplain fringing its right bank, that are hydrologically connected by pulses of flood and drought.

Ceratium *collection and morphological identification.*

We collected samples directly with a recipient (125 mL) from the sub-surface of the water column at the main channel and 6 floodplain waterbodies (Fig. 1; more information about sampling design in Devercelli et al. 2016) and fixed with acidified Lugol (1%) to perform the morphological identification of *Ceratium* (Nikon Eclipse TS100 microscope). For further *Ceratium* detection, we collected samples with 25 μm pore mesh (100-140 L). As a result, we found *C. furcoides* in the floodplain lake Ferranda and in Colastinecito de las Cruces Stream. Environmental characteristics of the ecosystems are detailed in Table S1 in the Supporting Information. The cells were fusiform and dorsiventrally flattened with a mean total length of 213.91 μm at the lake (SD ±16.03; range=179.36-

234.81; n=25) and 173.85 µm at the stream (±15.07; range=146.58-195.15; n=25). In its ventral view, specimens were 72.43 µm mean wide at the lake, and 53.06 µm at the stream; while cell biovolume was 42962.44 μm^3 (±12418.63) and 19741.61 μm^3 (±7013.66), respectively. We morphologically identified *C. furcoides* by its conical epitheca with a single horn and its short 4'triangular plate, which distinguished from *C. hirundinella* that has a bell-shaped epitheca and all apical horn plates reach the tip of the horn (Popovský and Pfiester 1990, Moestrup and Calado 2018). The hypovalve presented 2 or 3 horns that varied greatly in length (23.29-102.37 µm ±18.76).

Ceratium *phylogeny reconstruction.*

After morphological identification, we isolated cells from Colastinecito de las Cruces manually, washed them with TE solution buffer, and performed a pool of 15-30 individuals. Then, we performed genomic DNA extraction (InstaGene™ Matrix Bio-Rad). The V4 hypervariable region of the 18S rRNA gene was amplified by PCR using the eukaryotic primers 18S-408F and 18S-987R (Kok et al. 2012). PCR program was 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and an elongation step of 10 min at 72°C. After 1% agarose gel electrophoresis, amplicons were cut from the gel, purified with GENECLEAN® Turbo kit. and sequenced with Sanger (Macrogen Inc.). We obtained a 499 bp-length 18S rRNA partial sequence, which was uploaded to GenBank under the accession number MK567886. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis showed that *Ceratium furcoides* MK567886 sequence shares 100% identity with the two available sequences in databases (JQ639758, JQ639757). We performed the phylogenetic reconstruction for *Ceratium* using the Sanger sequence obtained in this work and the available sequences (GenBank) of the 18S rRNA gene of the genera *Ceratium, Tripos* and *Protoceratium* as an outgroup. The sequences were aligned using MAFFT v.7 (Katoh and Standley 2013), and the phylogeny was performed using the maximum likelihood method implemented in RAxML-HPC2 v8.2.10 (Stamatakis, 2014) with TrN+G4 evolutionary model, previously selected using moredeltest-ng v0.1.5 (https://github.com/ddarriba/modeltest). The resulting phylogenetic tree showed two highly supported clades (bootstrap >90%), one of them belonging to marine species and the other, to freshwater species (Fig. 2A). This is congruent with the recent separation of some marine species based on molecular and morphological features: freshwater species with six cingular plates remain as

Ceratium genus*,* whereas the marine species with five cingular plates were assigned into the genus *Tripos* (Gomez 2013). Within the freshwater clade, a first node defined a European clade containing *C. hirundinella*; then a second node gave rise to a clade containing American and Asian *Ceratium* sequences with two distinguished groups: one including uncultured *Ceratium*-like sequences from North America; and the other harboring the Paraná sequence obtained here together with Asian *C. furcoides,* which formed a highly supported monophyletic clade (100% bootstrap value). Accordingly, *C. furcoides* of Paraná floodplain were identical to Asian sequences and seems to be more related to its North American counterparts than to the European ones. This coincides with the first record found in North America that dates from late 1980s (Häkansson and Kling 1989), while in Europe it dates back to early 1940s (Heaney et al. 1988). Hence, tree topology (Fig. 2A) suggests a geographic pattern of *Ceratium* spp. with a closer origin for Asian and South American populations.

Notably, sequences from *Ceratium furcoides* and *C. hirundinella* did not show a clear phylogenetic segregation. We attribute this to the probable misidentification of species, but also is possible that the phylogenetic information contained in the ribosomal gene does not allow the separation of both species.

Ceratium furcoides *detection by HTS.*

Once the *Ceratium furcoides* population from Colastinecito de las Cruces Stream was characterized, a further survey of *Ceratium* sequences was conducted in the other ecosystems. We performed Illumina MiSeq sequencing (2x300 bp house in Biogenouest Genomics Genomer platform) from water samples filtered (100 to 150 mL) through 0.22 µm pore-size. The DNA extraction and PCR were performed as Metz et al. (2019) protocol. We defined operational taxonomic units as amplicon sequence variants (ASVs), by DADA2 v1.11. (Callahan et al. 2017). As a result, three ASVs were identified as *Ceratium* (sensu PR2 4.11 database using USEARCH 10; Edgar 2010). More details in Table S2 in the Supporting Information. To infer their phylogenetic position, we incorporated them by parsimony into the phylogenetic tree obtained above (PaPaRa v2.5; Berger and Stamatakis 2011). All the sequences clustered into the *C. furcoides* clade, corroborating the taxonomic affiliation (Fig. 2B).

ASV_2430 retrieved from Ferranda Lake showed 100% identity with *Ceratium furcoides* MK567886 sequence, whereas ASV_4869 and ASV_2996 retrieved from the main channel presented

98.8% and 99.1% identity, respectively. Thus, sequence identity was higher between organisms of floodplain waterbodies in comparison with the main channel, suggesting the presence of different populations that could have different environmental preferences. However, we cannot rule out the possibility that both ASVs correspond to pseudogenes of the same organism, meaning that they could be non-functional copies of the 18S rRNA gene that have been mutated over the course of evolution (Tutar 2012).

The obtained results highlight the relevance of combining classical morphological detection and characterization methods with next generation sequencing approaches, which thanks to its depth, allows the recovery of low frequency genetic variants and alert a potential invasion in its early stages. In conclusion, we reported for the first time a phylogenetic analysis of *Ceratium furcoides* from South America. Further ecological and genomic studies combining the sequencing of other genes (Zhang et al. 2005), would allow us to understand the processes accounting for the invasion success of *Ceratium* and tracing its dispersal route.

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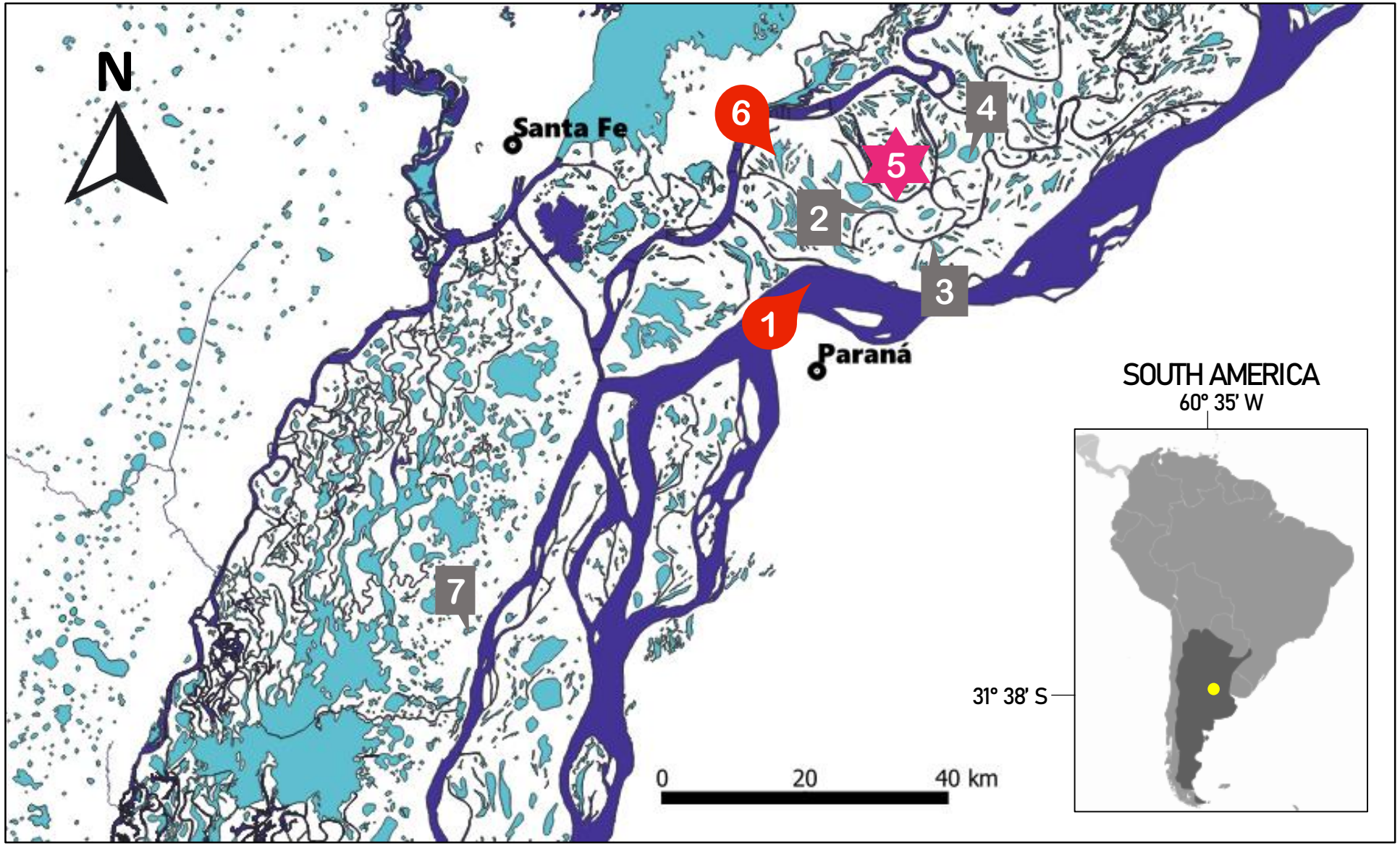
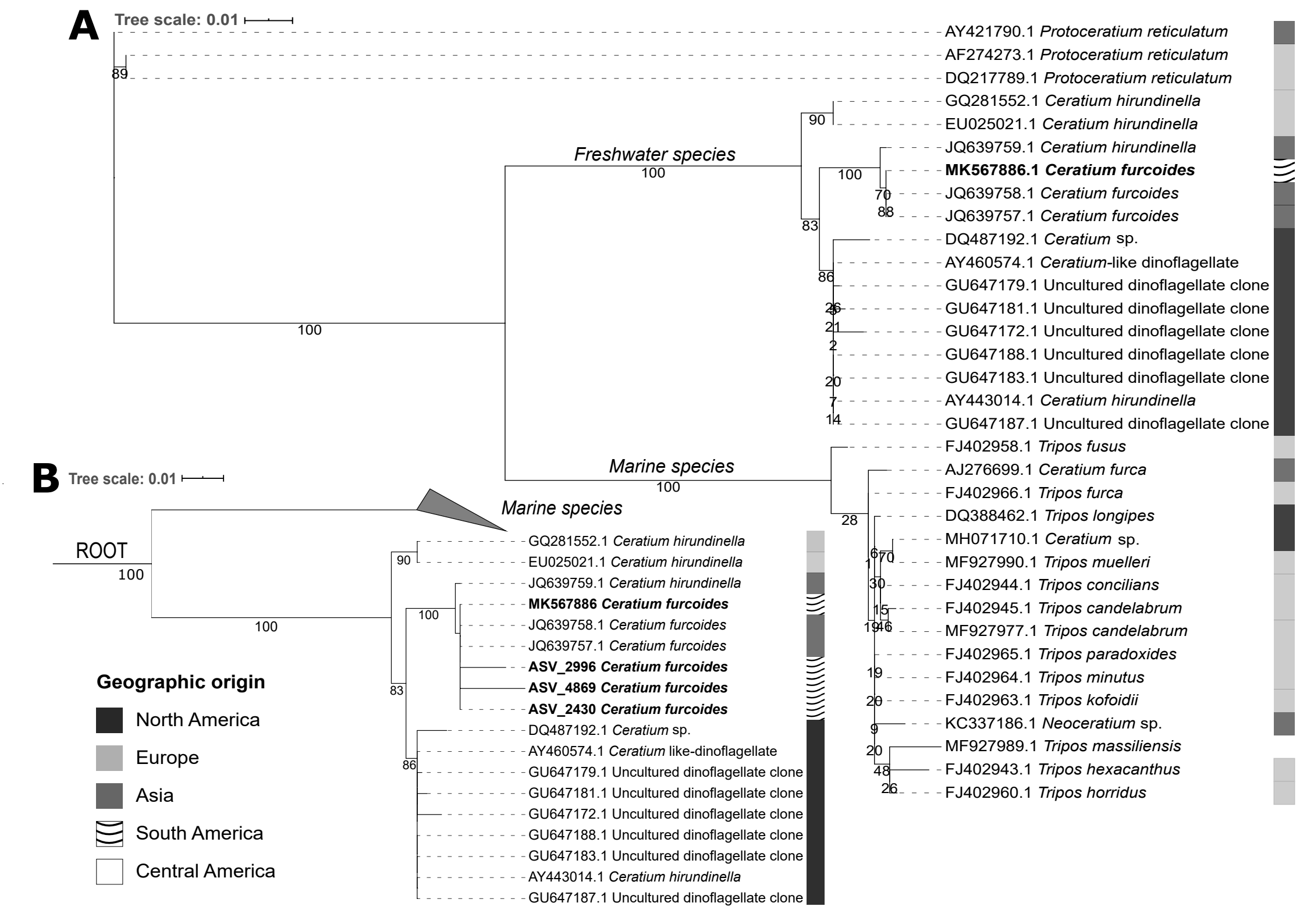
Fig. 1. Paraná fluvial system indicating the sampling sites localization at the main channel (1) and floodplain environments: Correntoso Stream (2); Correntoso Pond (3); Del Medio Lake (4); Colastinecito de las Cruces Stream (5); Ferranda Lake (6); Sauces Lake (7) (March 2014, September 2015, March 2016). Records of *Ceratium furcoides* 18S ribosomal gene are shown with color and shape: pink star for Sanger sequencing and red circles for Illumina MiSeq. The map was performed with the software Qgis 3.4 "Madeira".

Fig. 2 A. Phylogenetic tree based on the 18S ribosomal gene constructed using 33 sequences of *Ceratium* and *Tripos* retrieved from GenBank Database (min length 475 bp), and the *Ceratium furcoides* sequence obtained in this work (MK567886, in bold letter) by Sanger. Three *Protoceratium* sequences were used as outgroup. The access numbers of GenBank is shown in the tree. The bootstrap values are found in the nodes (1000 bootstrap repeats). The geographic origin of the sequences (colored squares), and the marine and freshwater species are indicated. **B**. Phylogenetic tree with Illumina sequences (in bold) incorporated by parsimony. Both trees were edited using the Interactive Tree of Life (http://itol.embl.de).

Table S1. Main characteristics of the sampled environments from the Paraná fluvial system. They were selected in order to encompass different types of ecosystems and hydrological connections that are characteristic of the fluvial system. Geographic coordinates, and range of variation of environmental variables are indicated at each sampling site: water temperature (Temperature), water transparency (Secchi), pH, conductivity, and percentage of saturation of dissolved oxygen (DO).

Table S2. Total number of sequence reads and ASV reads identified as *Ceratium,* and number of ASVs per sampling site (Illumina MiSeq).


N
Santa Fe
Paraná
1
2
3
4
5
6
7
0
20
40 km
SOUTH AMERICA
60° 35' W
31° 38' S


A
Tree scale: 0.01
AY421790.1 Protoceratium reticulatum
AF274273.1 Protoceratium reticulatum
DQ217789.1 Protoceratium reticulatum
GQ281552.1 Ceratium hirundinella
EU025021.1 Ceratium hirundinella
JQ639759.1 Ceratium hirundinella
MK567886.1 Ceratium furcoides
JQ639758.1 Ceratium furcoides
JQ639757.1 Ceratium furcoides
DQ487192.1 Ceratium sp.
AY460574.1 Ceratium-like dinoflagellate
GU647179.1 Uncultured dinoflagellate clone
GU647181.1 Uncultured dinoflagellate clone
GU647172.1 Uncultured dinoflagellate clone
GU647188.1 Uncultured dinoflagellate clone
GU647183.1 Uncultured dinoflagellate clone
AY443014.1 Ceratium hirundinella
GU647187.1 Uncultured dinoflagellate clone
FJ402958.1 Tripos fusus
AJ276699.1 Ceratium furca
FJ402966.1 Tripos furca
DQ388462.1 Tripos longipes
MH071710.1 Ceratium sp.
MF927990.1 Tripos muelleri
FJ402944.1 Tripos concilians
FJ402945.1 Tripos candelabrum
MF927977.1 Tripos candelabrum
FJ402965.1 Tripos paradoxides
FJ402964.1 Tripos minutus
FJ402963.1 Tripos kofoidii
KC337186.1 Neoceratium sp.
MF927989.1 Tripos massiliensis
FJ402943.1 Tripos hexacanthus
FJ402960.1 Tripos horridus
Freshwater species
100
Marine species
100
89
100
90
100
70
88
83
86
26
21
2
20
7
14
28
67
1
30
15
19
46
19
20
9
20
48
26
B
Tree scale: 0.01
ROOT
100
100
Marine species
GQ281552.1 Ceratium hirundinella
EU025021.1 Ceratium hirundinella
JQ639759.1 Ceratium hirundinella
MK567886 Ceratium furcoides
JQ639758.1 Ceratium furcoides
JQ639757.1 Ceratium furcoides
ASV_2996 Ceratium furcoides
ASV_4869 Ceratium furcoides
ASV_2430 Ceratium furcoides
DQ487192.1 Ceratium sp.
AY460574.1 Ceratium like-dinoflagellate
GU647179.1 Uncultured dinoflagellate clone
GU647181.1 Uncultured dinoflagellate clone
GU647172.1 Uncultured dinoflagellate clone
GU647188.1 Uncultured dinoflagellate clone
GU647183.1 Uncultured dinoflagellate clone
AY443014.1 Ceratium hirundinella
GU647187.1 Uncultured dinoflagellate clone
90
100
83
86
Geographic origin
North America
Europe
Asia
South America
Central America