Molecular insight reveals broad-scale spatial patterns in floodplain ciliate assemblages, whereas morphology reflects local environmental controls

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Abstract: One of the major goals in microbial ecology is to understand whether the empirical biogeographic patterns of macroorganisms also apply to microorganisms. Here, we used morphological data from live organisms, along with molecular data, to investigate the importance of spatial factors and environmental variables in influencing ciliate composition from floodplain lakes. Our main goal was to use 2 different approaches (morphological and molecular) to compare ciliate diversity and distribution patterns as well as to compare how these methods differ in their ability to detect distribution patterns and the roles of spatial and environmental factors that shape ciliate assemblages in the 4 largest floodplains in Brazil. Planktonic water samples were gathered from 33 lakes associated with 4 different river floodplain systems in Brazil. We analyzed ciliates in vivo and sequenced surface water DNA using a metabarcoding approach with general eukaryotic primers. We showed that the diversity of operational taxonomic units was much higher than that of morphospecies. Regardless of the method of identification, we found a consistent spatial assembly pattern of ciliate assemblages across the 4 floodplain systems. We also found that environmental filters had a stronger association with the morphological than with the molecular site-by-site dissimilarities. Meanwhile, biogeographic factors and the distance among sites limited the distribution of molecular-based composition, resulting in strong differences among the floodplain lakes analyzed. This finding suggests that ecological research and biomonitoring activities should find an equilibrium between morphological and molecular approaches because each approach provides unique insights.

Key words: microbial biogeography, protist, species sorting, dispersal limitation, metacommunity, freshwater

One of the main questions in ecology pertains to the distribution of species. Since the 18th century, many studies have been trying to understand how organisms are distributed in space and time (Leibold et al. 2004), addressing the biogeographic patterns of factors such as speciation, extinction, dispersal events, and environmental constrains (Heino et al. 2017). Because the core of theoretical ecology has largely been applied to plants and animals, applying these concepts to microbial communities is challenging. The primary gaps between traditional and microbial approaches stem from

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differences in scale and physiologies between macro and microorganisms (Martiny et al. 2006) as well as among types of microorganisms (van der Gast 2015).

There exists a viewpoint that microorganisms have such high dispersal rates that they are not restricted by geographic barriers. This perspective supports the traditional hypothesis proposed by Baas-Becking (1934) that "everything is everywhere, but the environment selects" (p. 15). Baas-Becking's main argument presumes that local environmental conditions and biotic interactions, such as competition and predation, are the most important factors determining local composition of species. Microorganisms are small, numerous, and have high population densities (Fenchel and Finlay 2004). In addition to their ability to produce cysts, this combination makes them less prone than macroorganisms to local extinction and increases their potential for dispersal events (Astorga et al. 2012). However, more recent biogeography perspectives suggest that some microbial taxa may be endemic (van der Gast 2015, Tessler et al. 2017) and that most protistan taxa may be geographically limited (Lentendu et al. 2019). Community assembly mechanisms involve historical and evolutionary factors, which, together with dispersal limitations, may restrict species occurrences at different locations (Heino et al. 2017). One question that remains is whether the empirical biogeographic patterns of macroorganisms also apply to microorganisms (Shoemaker et al. 2017).

Microbial ecologists face challenges in testing ecological theory at such a small scale (Prosser et al. 2007). Attempting to apply traditional methods in ecological studies on microbial eukaryotes (i.e., protists) presents some problematic issues. For instance, morphological analyses are frequently limited because some specimens are injured during fixation, the presence of cryptic and polymorphic species confound diversity assessments, and there are only a few specialized identification professionals (Sáez and Lozano 2005). There is also a bias toward identifying only the most abundant species, thus ignoring most of the rare ones (Dunthorn et al. 2014). However, modern sequencing techniques offer new potential to largely overcome the limitations of traditional microbial methods for accurately estimating protist diversity, which has enhanced our ability to study microorganisms as compared with research from 3 to 4 decades in the past (Lentendu et al. 2018, Pitsch et al. 2019).

High-throughput sequencing (HTS) technologies are capable of generating hundreds of millions of amplicons, an optimal method for thorough assessment of the diversity of complex microbial assemblages (Mahé et al. 2015a, Lentendu et al. 2018). This novel strategy has also revealed thousands of rare species that could not be detected under microscopy or Sanger's sequencing (Dunthorn et al. 2014, Logares et al. 2015). HTS data are now fundamental for ecological studies of microbial organisms, especially those investigating biogeographic patterns (van der Gast 2015). For instance, at broad spatial scales, the finer taxonomic resolution of the molecular approach compared with the morphological approach could provide evidence for greater assemblage variation across regions. In contrast, at regional scales, the higher capacity of the molecular approach to detect rare species may show higher among-site assemblage similarity, which could weaken the ability to detect the effects of environmental filtering on species occurrence (Leibold et al. 2004). Moreover, molecular approaches do not ascertain whether the operational taxonomic units (OTUs) found are active microorganisms or encysted (i.e., dormant) stages and, therefore, whether they have a role in ecosystem functioning. As a consequence, the importance of local environmental conditions for structuring microbial assemblages at regional scales should be more easily detected through morphological surveys.

Among microorganisms, ciliates can be found almost everywhere on Earth (Foissner et al. 2008). Ciliates are considered an essential component of microbial food webs because they feed on bacteria, cyanobacteria, phytoplankton, and other protists (Meira et al. 2018) while being eaten by microcrustaceans and rotifers (Arndt 1993). Moreover, particular ciliate traits, such as high sensitivity to physical and chemical alterations and their preference for a variety of occupied niches, make ciliates good indicators of ecosystem health (e.g., Segovia et al. 2016). Many studies focusing on ciliates have recently been published because of their relevance for ecosystem functioning and stability (Stoeck et al. 2014). However, most studies investigated ciliate assemblages in marine (e.g., de Vargas et al. 2015) and soil (e.g., Forster et al. 2016) environments, whereas only a few have assessed ecological aspects of the microbial eukaryotic assemblages in freshwater systems (Gran-Stadniczeñko et al. 2019, Pitsch et al. 2019), especially in Neotropical environments (but see Lentendu et al. 2019).

Here, we used morphological data from live organisms and molecular data from HTS to investigate the relative importance of spatial and environmental variables in influencing ciliate assemblage composition in floodplain lakes. Our main goal was to use 2 different approaches (morphological and molecular) to compare ciliate diversity and distribution patterns as well as to compare how these methods differ in their ability to detect species distributions and the roles of spatial and environmental factors that shape ciliate assemblages in the 4 largest floodplains in Brazil: Upper Paraná River, Pantanal, Araguaia, and Amazônia. We formulated the following hypotheses: H1) differences in species composition among floodplains (broad scale) will be found for both approaches; H2) ciliate composition dissimilarity (i.e., beta diversity) within each floodplain (regional scale) will be greater for the morphological compared with the molecular approach because we expect the molecular approach to identify more rare species common to multiple sites, thereby masking the effects of environmental filtering; and H3) the morphological approach will be more sensitive to detecting associations of environmental variables with ciliate

assemblage composition, whereas the molecular approach will be more sensitive to detecting the effects of broad-scale spatial processes.

METHODS

To answer our research questions, we conducted a field study in floodplain lakes and contrasted 2 approaches, morphological and molecular, to assess the importance of spatial factors and environmental variables in shaping ciliate assemblages. We gathered planktonic water samples from 33 lakes in 4 different river-floodplain systems in Brazil. We analyzed ciliates in vivo and sequenced surface water DNA using a metabarcoding approach with general eukaryotic primers. We performed permutational multivariate analysis of variance (PERMANOVA) to verify spatial changes among floodplains in the composition of ciliate assemblages for both morphological and molecular data and a permutational analysis of multivariate dispersions (PERMDISP) to evaluate the degree to which ciliate assemblages differed within floodplains. Moreover, to evaluate the role of the environment and dispersal potential on the composition of ciliate assemblages, we used variation partitioning with distance-based redundancy analysis (dbRDA), including both abiotic (physical and chemical) and biotic (food resources and potential predators) factors. Details about all methodological steps are described below.

Study area and sampling design

Our study was conducted on the 4 largest Brazilian river-floodplain systems: Upper Paraná River (Paraná, Baía, and Ivinheima rivers), Pantanal (Miranda and Paraguai rivers), Araguaia (Araguaia River), and Amazônia (Solimões and Amazon rivers) in Brazil (Fig. 1). These 4 ecosystems support a high biodiversity of organisms, based on periods of periodical flooding during the rainy season of the southern hemisphere (Junk et al. 1989). We sampled in 33 connected lakes between August 2011 and May 2012 (Upper Paraná: $n = 11$; Pantanal: $n = 11$; Araguaia: $n = 6$; Amazônia: $n = 5$). We

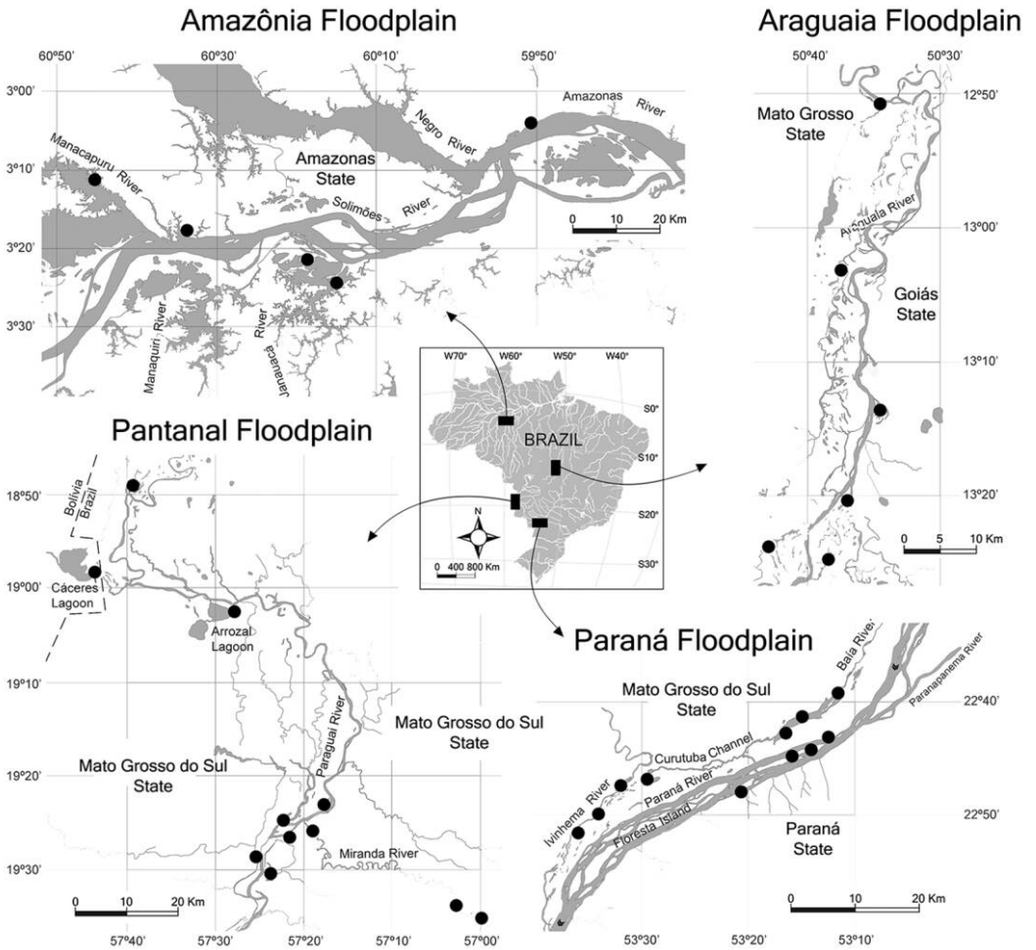

Figure 1. Map of the study area showing the location of all surveyed lakes (black dots) within each of the 4 largest Brazilian floodplains: Amazônia, Araguaia, Pantanal, and Paraná.

sampled each lake once, and we conducted all sampling campaigns in the dry season except the Paraná floodplain where, even though sampling took place in the rainy season, there was no flood pulse in the Paraná River that year.

The Upper Paraná River has a large anastomosed channel, a wide alluvial plain reaching ≤20 km in width, and a drainage area of 891,000 km^2. It is the most extensively impounded of the 4 studied floodplains with more than 130 dams. These dams retain high amounts of nutrients and sediments, yielding a completely modified hydrology with oligotrophic waters in the main channel (Roberto et al. 2009). It varies in types of habitats, among them numerous secondary channels, lakes, and 2 main tributary rivers: Baía and Ivinheima.

The Pantanal biome is characterized by its very high biological productivity and, consequently, by substantial biodiversity, being one of the world's largest wetlands (140,000 km^2 area). It is a heterogeneous ecosystem with ≥10 subregions with differing geomorphological and ecological characteristics, which creates idiosyncrasies in the occurrence, duration, and intensity of flooding depending on the subregion. Major flooding in the northernmost region coincides with the rainy season, but there is a delay (~40 d) before the flood reaches the southern region through the main tributaries, streams, and non-channelized flow paths (Junk et al. 2006).

The Araguaia River has one of the largest South American basins with an average flow of 6430 m^3/s. The Middle Araguaia River, located in eastern Amazônia, is 1160 km in length with a drainage area of 320,290 km^2 (Lininger and Latrubesse 2016). It is formed by a well-developed floodplain, with much of its area located in the ecoregion locally known as Cerrado (Brazilian savannah), a biodiversity hotspot. In this floodplain, flooding occurs between November and April, and drought conditions extend from May to October (Latrubesse and Stevaux 2002).

The Amazon River is the world's greatest river both in extent and water flow. Wetlands from the alluvial floodplain of the Amazon River and primary tributaries cover >300,000 km^2 (Junk 1997). The Brazilian Amazonia floodplain comprises the confluence of the Solimoes and Negro rivers to form the Amazon River. The floodplain's wetland habitats have high spatial heterogeneity, providing conditions for the establishment and development of several biological communities, which results in enormous biodiversity. The water level can rise ≤10 m a.s.l. on average during floods. Usually, flooding begins in November and reaches its maximum level in July. The dry period starts in August, when the water runs from the lakes to the rivers, with October as the driest month.

Sampling and morphological identification of ciliates

We analyzed ciliates in vivo (Madoni 1984). At the subsurface (~20 cm below the water–air interface) of the limnetic region of each lake, we collected 2 L of water in polyethylene plastic bottles, which were stored in coolers and transported by boat to the laboratory for analysis within 6 h of sampling. We filtered this water through a 5-μm-mesh net into a 100-mL concentrated sample. We then homogenized the samples by mixing them to avoid biases in species richness within the flask. From each 100-mL sample (i.e., each lake), we analyzed ten 1-mL subsamples. We identified ciliates to the lowest taxonomic level possible (morphospecies) with a CX-41 optical microscope (Olympus, Tokyo, Japan) at 100 to 400× magnification following Foissner et al. (1999) and Berger and Foissner (2014). This method allows for a good inventory of ciliate composition in each locality when compared with staining techniques because species are lost in fixation and impregnation processes (Madoni 1984). However, we recognize that the accuracy of species identification through live counting is limited (Pitsch et al. 2019).

DNA extraction and amplification

To obtain the DNA extraction and amplification, we first filtered ~1.5 L of lake water (collected and stored following the same protocol as described above) through 13-mm diameter Nuclepore™ polycarbonate membranes with 0.8-μm pore size (Whatman, Maidstone, United Kingdom) and stored samples at 20°C. We then placed filters in 25-mL centrifuge eppendorfs (Eppendorf, Hamburg, Germany) and extracted DNA with DNeasy® Blood and Tissue kits (Qiagen, Hilden, Germany). We amplified extracted DNA with general eukaryotic primers that targeted the V3 hypervariable region of the 18S-rRNA locus following Nolte et al. (2010). We then sequenced the amplified products with a MiSeq™ FGx™ sequencer (Illumina®, San Diego, California). The raw sequences were deposited at the European Nucleotide Archive's Sequence Read Archive and are publicly available under the BioProject PRJEB26716 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJEB26716).

Bioinformatics

To proceed with bioinformatics and gather molecular data, we first used Swarm (version 2; Mahé et al. 2015b) to process the sequences for OTU clustering as described by Lentendu et al. (2019). We filtered assembled paired-end reads and retained them if they contained both primers (minimum overlap set to $^2/_3$ the primer length), met a minimum length of 90 nucleotides, and had no ambiguous positions. Then, we used VSEARCH (Rognes et al. 2016) with the Protist Ribosomal Reference database for taxonomic assignment (Guillou et al. 2012). We assigned amplicons to their best hit or co-best hits, in which case we used the least-common ancestor with an 80% consensus threshold to resolve the taxonomy. For further analysis we only considered OTUs assigned to Alveolata, a recognized successful lineage of ciliates (Foissner et al. 2008). For more details regarding the bioinformatics used here please see Lentendu et al. (2019).

Environmental filtering

We considered key environmental variables that are known to shape the composition of ciliate assemblages, including both abiotic (physical and chemical) and biotic (food resources and potential predators) factors (Segovia et al. 2017). We used a YSI 550A handheld instrument (Yellow Springs Instruments, Yellow Springs, Ohio) to measure dissolved O (mg/L) and temperature (°C), a DM-2 thermostatic vessel (Digimed Analytical Instrumentation, São Paulo, Brazil) to measure conductivity (μS/m) and pH, a portable turbidimeter (LaMotte®, Chestertown, Maryland) to measure turbidity (NTU), and a Secchi disk to measure water transparency (cm). We collected 2-L water samples from the pelagic region (~20 cm below the water–air interface) with polyethylene plastic bottles for further quantification of N and P concentrations in the laboratory. The samples were preserved in an ice cooler and transported by boat to the laboratory. We stored samples in acid-washed polyethylene flasks and at –20°C for a maximum of 2 mo. In the lab we determined total N, NO_3^-, NH_3, total P, and P concentrations (μg/L) with an ultraviolet spectrophotometer (UV-Vis Shumadzu 2600i) according to Roberto et al. (2009).

Along with samples for ciliates and abiotic measurements, we also collected water samples to quantify bacteria, flagellates, zooplankton, and phytoplankton. These groups were included in our analyses as components of the environmental filter that shapes the assemblage of ciliates in each environment (Segovia et al. 2017, Meira et al. 2018). We sampled water for analyses of microbial assemblages with acid-washed polyethylene flasks (175 mL) at the subsurface (~20 cm below the air–water interface) at the central, deepest region of each lake. For zooplankton analyses, we used a pump and a plankton net (68-μm mesh) to filter 600 L of water.

Bacteria and heterotrophic flagellate samples were fixed in situ with alkaline Lugol's solution, borate buffered formalin, and sodium thiosulfate (Sherr and Sherr 1993), and stored them in an ace cooler for transport from the boat to the laboratory. Within 24 h after sampling, we filtered the samples through black Nuclepore filters (0.2 μm for bacteria and 0.8 μm for heterotrophic flagellates), stained them with fluorochrome DAPI (4',6-diamidino-2-phenylindole; Porter and Feig 1980), and stored them at –20°C for a maximum of 2 mo. We quantified bacteria and flagellates with an epifluorescence microscope at a magnification of 1000× (Olympus BX51). We preserved phytoplankton samples with acidified Lugol's solution and identified and quantified them using an inverted microscope (Olympus CKX 41), according to Utermöhl (1958). We quantified and identified zooplankton (rotifers, cladocerans, and copepods) according to Lansac-Tôha et al. (2009) by counting ≥3 subsamples under an optical microscope (Olympus CX41) at 40 to 400× magnification depending on the taxonomic group.

We used both abiotic and biotic predictor variables to explain differences in ciliate assemblage composition within and among floodplains. For biotic predictors we considered the density of bacteria and flagellates (ind./mL), as well as assemblage structure of zooplankton and phytoplankton. To summarize the multi-taxa composition of zooplankton and phytoplankton assemblages, we used the first 2 axes from a principal coordinates analysis (PCoA; Bray–Curtis distances) applied separately on each group (cmdscale function in the *stats* package in R version 4.0.3; R Project for Statistical Computing, Vienna, Austria). These composite variables account for potential interactions among these biological groups and have been considered as fundamental for unraveling the role of environmental filters on spatial processes (Brown et al. 2017). To visualize the variation of all abiotic and biotic predictor variables among the 4 floodplain systems, we performed a principal component analysis with the prcomp function in the *stats* package (Fig. S1).

Data analysis

H1: Contrasting approaches on broad-scale data: Implications for variation in species composition To test for spatial differences in broad-scale (i.e., between floodplain) composition of ciliate assemblages for both morphological and molecular data (H1), we performed a PERMANOVA (9999 permutations; adonis function of the *vegan* package, version 2.5-7; Oksanen et al. 2018), controlling the identity of each floodplain as a factor. When the difference among all floodplains had a $p < 0.05$, we used pairwise tests to detect differences between each pair of floodplains and adjusted p-values for pairwise tests with the Bonferroni correction. We performed PERMANOVAs on Jaccard dissimilarity data (presence–absence data) to best compare between morphological and molecular data.

H2: Contrasting approaches on regional data: Implications for beta diversity To estimate differences in the degree to which ciliate assemblages differed within floodplains (regional scale; H2), we performed a PERMDISP (Anderson et al. 2006) using the betadisper function in the *vegan* package (type = centroid). This test is based on the average dissimilarities from each sample to the centroid of its group in a multivariate space built using PCoA (Anderson et al. 2006). Thus, higher variation in assemblage composition across sites (i.e., beta diversity) is depicted by greater distances to a group's centroid (Anderson et al. 2006). Four groups were considered representing each individual floodplain (Amazônia, Araguaia, Pantanal, and Paraná). We performed ordinations on dissimilarity matrices generated using the Jaccard index, calculated from site-by-species (or OTUs) presence–absence data. Finally, the distances of sites (i.e., lakes) to their corresponding centroids (i.e., floodplains) were used to assess differences between morphological and molecular data using a 2-way ANOVA (aov function in the *stats* package), also controlling for the floodplain to which they belong and an interaction term between floodplain and approach. We then used Tukey's honestly significant

difference post-hoc tests to identify differences in dissimilarities between floodplains.

H3: Environmental and spatial covariates of alternative approaches To evaluate the role of the environment and dispersal potential on the composition of ciliate assemblages (H3), we used variation partitioning with dbRDA (Legendre and Andersson 1999) with the Jaccard dissimilarity matrices, calculated separately for morphological and molecular approaches, as response variables. For explanatory matrices we used environmental data (E), and 2 different models considering different spatial extents. For the fine-scale spatial model (F) we used a method developed by Declerck et al. (2011), which is suitable for sampling designs where basins are located far from each other, as was the case in our study. This approach is based on distance-based Moran's eigenvector maps (dbMEM), which is a spatial analysis method derived from Cartesian geographical coordinates, in this case the distance among studied lakes. We used dbMEMs as explanatory variables in the fine-scale spatial model. We used the function create.MEM.model from the *adespatial* package (version 0.3-14; Dray et al. 2018) to create a staggered matrix of spatial variables such that each block represented the spatial structure of lakes from the same floodplain, whereas lakes from other floodplains were assigned a value of 0. To construct the broad-scale spatial component (B), we used a dummy variable representing floodplain identity (among floodplains) because MEMs perform poorly when there are large gaps among regions, such as those of the studied floodplains (Declerck et al. 2011).

To select the best set of local environmental features and fine- and broad-scale explanatory variables associated with the variation in ciliate assemblages, we used the ordiR2step function in the *vegan* package to carry out a forward selection procedure designed for constrained ordination methods with 2 stopping rules (Blanchet et al. 2008). We did this to prevent artificially inflated explanatory powers of our constrained ordination models (i.e., model overfitting). Forward selection assumes that there is evidence to suggest that all variables affect the assemblage composition and proceeds only if the global model (i.e., including all predictor variables), which is tested 1st, explains a high proportion of the total variance. The 1st stopping rule entails the adjusted R^2-value of the reduced model exceeding that of the critical p-value (at $\alpha = 0.05$). The 2nd rule is related to the comparison of adjusted R^2-value explained by the selected variables to adjusted R^2-value explained by the global model, stopping when the adjusted R^2-value starts to decay. Before statistical analyses, we checked for multicollinearity in the environmental matrix by computing the variance inflation factors (VIFs), and we removed variables for which the variance of a regression coefficient was inflated in the presence of another explanatory variable (i.e., VIF > 5; Borcard et al. 2018).

We decomposed the variation in the composition of ciliate assemblages into purely environmental (E | F + B), purely fine-scale spatial extent (F | E + B), and purely broad-scale spatial extent (B | E + F) components, as well as their shared fractions. We estimated the proportion of explained variance of each component through adjusted coefficients of determination (adjusted R^2; Peres-Neto et al. 2006). The obtained p-value for each pure component was calculated based on 999 permutations (Peres-Neto et al. 2006). We built the dbRDA with the capscale function and ran the variation partitioning with the varpart function, both from the *vegan* package.

RESULTS

Ciliate diversity and composition

We registered a total of 69 morphospecies and 1038 OTUs of ciliates with morphological and molecular approaches, respectively. For the morphological approach, Paraná (44) and Pantanal (38) showed greater species richness, compared with Amazônia (20) and Araguaia (18). Mean alpha diversity was more homogeneous within lakes among floodplains, with the highest value recorded for the Pantanal (10; Fig. 2). For the molecular approach, the highest OTU richness was recorded in the Pantanal (711) and the lowest in the Amazônia (345) floodplains. In terms of alpha diversity, the highest means occurred in the Pantanal (180) and Araguaia (150) rivers, whereas the Amazônia (130) and Paraná (110) rivers had the lowest number of lake-level OTUs (Fig. 2).

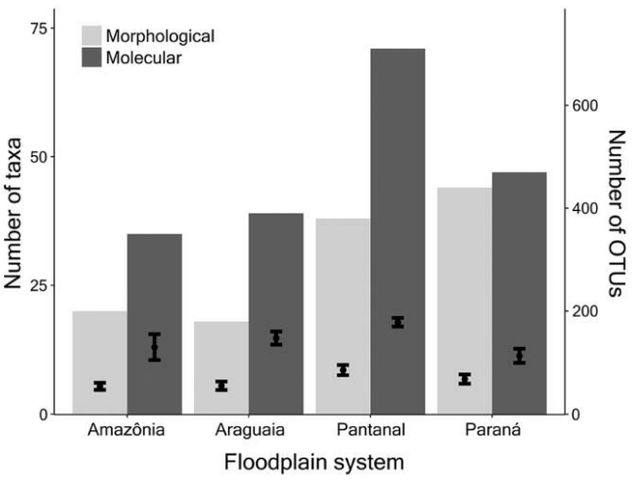

Figure 2. Diversity of protist ciliates in each of the 4 largest Brazilian floodplain systems (Amazônia, Araguaia, Pantanal, and Paraná) for the morphological (number of morphospecies) and molecular (number of operational taxonomic unit [OTUs]) approaches. Barplot represents total richness in each floodplain, and error bars depict the mean and SE for alpha diversity of lakes within floodplains.

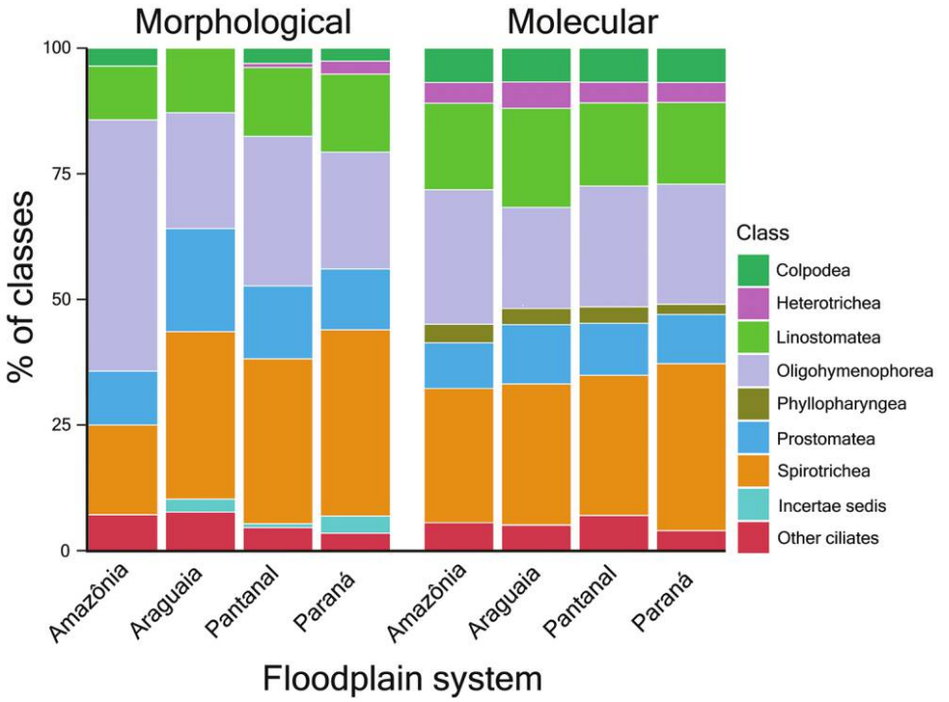

Figure 3. Relative contribution of classes to composition of ciliate assemblages for each floodplain system (Amazônia, Araguaia, Pantanal, and Paraná) in Brazil for morphological (number of species/class) and molecular (number of OTUs/class) approaches.

In general, the contribution of different classes of ciliates was similar between the morphological and molecular approaches (Fig. 3). There was a predominance of Oligohymenophorea and Spirotrichea for both approaches, with Spirotrichea's contribution being slightly higher in all floodplains except for the Amazônia floodplain, which showed a clear predominance of the Oligohymenophorea for the morphological approach (Fig. 3). The classes Litostomatea and Prostomatea were also common in all of the floodplains analyzed, highlighting the Araguaia floodplain, which registered the largest contribution of Prostomatea to the morphological approach and Litostomatea to the molecular approach. The Phyllopharyngea class was exclusively identified by the molecular approach, whereas ciliates belonging to incertae sedis were found only for the morphological approach (Fig. 3).

Considering the among-floodplain differences in ciliate composition (H1), the PCoA ordination suggested a clear distinction of each floodplain centroid group, especially for the molecular approach (Fig. 4B), although a higher overlap was found for the morphological approach (Fig. 4A). This pattern was further evidenced by the PERMANOVAs (main and pairwise tests), which revealed differences in ciliate composition among all floodplains based on both morphological and molecular approaches (Table S1).

Regarding the within-floodplain beta diversity (dissimilarity among lakes within floodplains; H2), the 2-way ANOVA indicated that lakes were more dissimilar (showed greater values of the distance to centroid; PERMDISP results) under the morphological than under the molecular approach (Table S2, Fig. 4C). Tukey's post-hoc tests also revealed that the among-floodplain dissimilarities were different only between the Paraná and Araguaia ecosystems, regardless of the approach (Table S2, Fig. 4C).

Relative importance of environmental and spatial factors

Variation partitioning results showed that the contributions of environmental and spatial factors in explaining assemblage composition differed between morphological and molecular approaches (H3; Table 1, Fig. 5A, B). According to the explanatory power (adjusted R^2), the morphological-based taxonomic composition of the ciliate assemblages showed a stronger relationship with the environmental conditions (17%) than that observed under the molecular-based approach (8%). Meanwhile, the broad-scale spatial component was more important for molecular-based (20%) than morphological-based assemblages (8%). In the same way, the fine-scale spatial component was more related to molecular-based (7%) than morphological-based (3%) assemblages. The shared components among all factors are given in Fig. 5A and B. However, a large amount of variation remained unexplained for both approaches.

Those environmental variables selected as the best set to explain the compositional patterns of ciliate assemblages varied depending on the approach (Table 1). For instance, bacterial density, dissolved O, PCoA1 of copepods, conductivity, water temperature, P, and PCoA1 of rotifers

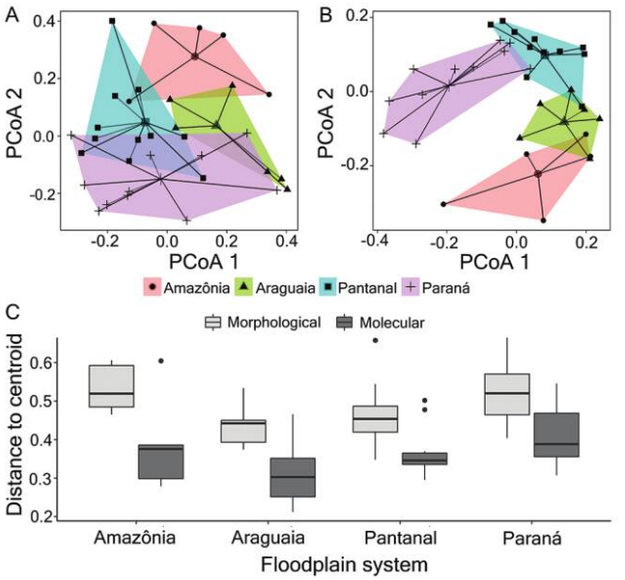

Figure 4. Dissimilarities registered at each of the 4 largest Brazilian floodplains systems (Amazônia, Araguaia, Pantanal, and Paraná). Principal coordinate plots derived from the morphological (A) and the molecular (B) data. Lines represent the distances between the sampling sites and the centroid of each group, as defined by the floodplain system. Boxplots of distances to centroid group for morphological and molecular approaches (C). The central line denotes the median value, the box denotes 25th and 75th percentiles, whiskers represent the smallest and largest value within 1.5× the interquartile range below and above percentiles, and dots indicate outliers.

were selected for the morphological approach, whereas bacterial density, dissolved O, PCoA1 of copepods, water temperature, and pH were selected for the molecular approach. Furthermore, out of the 7 derived dbMEMs generated, the fine-scale spatial component was composed of the same eigenvectors (dbMEM.2 and db.MEM.6) after forward selection for both morphological and molecular approaches.

DISCUSSION

Using a continent-wide sampling design, we studied 4 different biomes, each with unique habitats and environmental heterogeneities that provide optimal conditions for the establishment of different biological communities. Here, we used morphological data from live organisms and molecular data from HTS to compare how these methods differ in their ability to detect the distribution of ciliate morphospecies and the roles of spatial and environmental factors that influence ciliate assemblage composition in the 4 largest floodplains in Brazil. As expected, we found differences in species composition among the floodplains for both approaches and that ciliate composition dissimilarity (i.e., beta diversity) within each floodplain (regional scale) was greater for the morphological compared with the molecular approach. Moreover, we found striking differences in the underlying mechanisms of community assembly between morphological and molecular approaches. These findings suggest that ecological research and biomonitoring activities should find an equilibrium between morphological and molecular approaches because each approach provides unique insights.

Higher diversity using the molecular approach

We showed that the diversity of OTUs was much higher than that of morphospecies, although values were not consistent across floodplains. Indeed, genetic variation is usually higher than morphology-based estimators, especially because the correspondence between morphotypes and phylotypes is not always evident (Santoferrara et al. 2014). The differences between morphological- and molecular-based approaches may result, in part, from high polymorphism in small subunit ribosomal rDNA among species (Dunthorn et al. 2014). For instance, cryptic species (i.e., genetic species that converge into very close morphological resemblances) are widespread among eukaryotes and have been found in several freshwater ciliates (Sáez and Lozano 2005). Thus, for many protists, a morphology-based taxonomic description is quite restricted in terms of number of species (Boscaro et al. 2017).

Table 1. Total variance in ciliate assemblage dissimilarities explained by pure environmental (E), fine-scale spatial (F), and broad-scale spatial (B) components and their relative contributions (adjusted [adj.] R^2 and p-values), after variation partitioning analysis in distance-based redundancy analysis, for morphological and molecular high-throughput sequencing technology approaches.

	Morphological		Molecular	
Model component	Adj. R^2 (%)	p	Adj. R^2 (%)	p
E	25.4[a]	0.001	15.8[b]	0.001
F	3.40[c]	0.032	10.6[c]	0.001
B	17.1[d]	0.001	27.4[d]	0.001
Variation partitioning				
E|(F + B)	16.6	0.001	8.2	0.025
F|(E + B)	2.7	0.089	7.4	0.009
B|(E + F)	8.3	0.013	19.8	0.001

[a] E model constructed with the environmental variables bacteria density, dissolved O, copepod principal coordinate analysis axis 1 (PCoA1), conductivity, water temperature, P, and rotifer PCoA1.

[b] E model constructed with the environmental variables dissolved O, water temperature, bacteria density, copepod PCoA1, and pH.

[c] F model constructed with Moran's eigenvector maps variables dbMEM.2 and dbMEM.6.

[d] B dummy variable differentiating the floodplains.

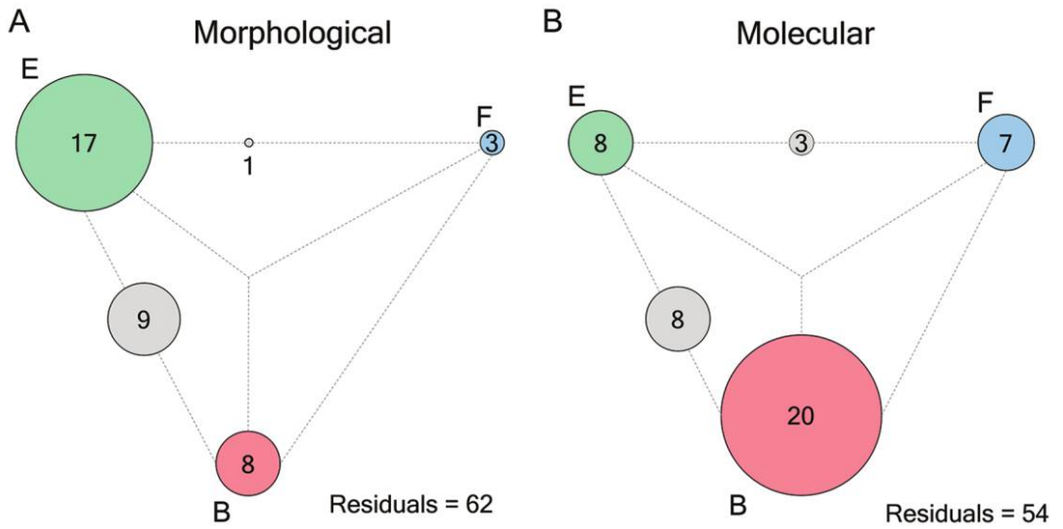

Figure 5. Variation partitioning analysis of morphological (A) and molecular (B) ciliate assemblages explained (values in circles = % adjusted R^2) by environmental component (E; green), fine-scale spatial component (F; blue), broad-scale spatial component (B; red), and their shared components (gray). Circle size is proportional to the respective percentages of explained variation.

Another major aspect to consider is the core definition of OTUs. In most cases, OTUs are defined based on a similarity threshold, so that all sequences that are more similar than a given threshold are grouped into the same OTU (Logares et al. 2015). Our OTUs were defined with a single linkage clustering algorithm, as this has been suitable for species-level distinctions of most protist groups (de Vargas et al. 2015, Mahé et al. 2015a). However, many OTUs matched reference sequences from the same species. For instance, we had *Tintinidium balechi* and *Vorticella aquadulcis* assigned to 27 and 15 different OTUs, respectively, whereas the morphological approach simply identified 2 species. Thus, we identified a delicate tradeoff between morphological and taxonomic molecular approaches, with the number of OTUs probably inflating the estimation of true species diversity. However, the opposite may also be true in that in-vivo morphological analysis may underestimate the real assemblage diversity.

Ciliate composition is different among floodplains, regardless of the approach (H1)

Regardless of the method of identification, we found a consistent spatial assembly pattern of ciliate assemblages across the 4 floodplain systems. This finding agrees with the growing body of evidence that protists may show biogeographical patterns that resemble those of macroorganisms, which are based on dispersal constraints (Martiny et al. 2006, Fontaneto and Hortal 2013). Greater geographic distance makes communities more dissimilar because of lower dispersal rates, which maintains spatial variation in community composition (Soininen et al. 2011). Biogeographical processes that may influence the current distribution of organisms include dispersal, past and contemporary environmental filtering, and ecological drift, which may result in compositional variation among communities related to morphology or molecular features (Heino et al. 2017).

Specifically for our sample design, the biogeographic patterns and differences in species composition among the floodplains may be due to a series of factors, such as the lack of hydrological connectivity between the floodplains, reflecting dispersal limitation; climatic differences, ranging from tropical to subtropical climate, that may impose restrictions on species; differences in the biome in which each floodplain is located, where landscape variation is also accompanied by species turnover; and different anthropogenic pressures in each biome (e.g., the series of cascading reservoirs in the Paraná River floodplain), which alter environmental and ecosystem conditions and cause strong changes in species composition.

Within floodplain assemblage dissimilarity (i.e., beta diversity) is higher for the morphological approach (H2)

At fine-scales, high dispersal rates of organisms may interfere with distribution and beta diversity patterns of aquatic assemblages (Heino et al. 2015). This is likely true for microorganisms, in particular because they have high rates of replication and short life cycles and, by achieving high population sizes, can disperse at rates high enough to reduce assemblage dissimilarity (Lansac-Tôha et al. 2021). This is possible because, with high dispersal and subsequent continuous colonization, species can exist in habitats that are normally outside of their ecological niche (Brown et al. 2017). Here, in addition to evaluating this pattern for protist ciliates, we compared 2 different approaches, and as expected, the PERMDISP results showed that lakes within floodplains

were more similar under the molecular than under the morphological approach, i.e., the detection of homogenizing effects was greater in the molecular approach.

One of the main advantages of using the molecular approach is the record of many rare OTUs, which are often overlooked in studies based only on morphology (Dunthorn et al. 2014, Logares et al. 2015). Although the better detection of rare species by the molecular approach can lead to greater community variation at broad scales (see below), at fine scales this higher capacity may result in higher among-site assemblage similarity when compared with the morphological approach. In contrast, the occurrence of rare species is more sporadic for the morphological approach, which contributes to increased turnover between sites.

Environmental filtering is related to morphological ciliate data, whereas broad- and fine-scale spatial processes are related to molecular ciliate data (H3)

We used variation partitioning in constrained ordination analysis to infer the relative importance of niche- and dispersal-based dynamics for different assemblages (Leibold et al. 2010, Brown et al. 2017). When we controlled for the effects of environmental factors as well as fine- and broad-scale dispersal proxies, we found substantial differences in the contributions of environmental vs spatial drivers between morphological- and molecular-based approaches. These differences, resulting from the choice of approach, directly interfere with interpretation of the primary mechanisms structuring community assembly mechanism.

Traditional identification and description of microbial eukaryotes has been microscopy based, which led some researchers to conclude that global protist diversity may be relatively low (Fenchel and Finlay 2004). Our morphological results partially support this assumption. However, our molecular-based results show a major role of the broad-scale spatial factor (among floodplains) in explaining variation, which leads us to speculate that ciliate assemblages may be strongly spatially structured. The importance of broad-scale patterns for assemblage composition suggests that biogeographic factors and the distance among sites limit the dispersal of ciliates, at least at large scales, resulting in different species composition between the floodplains analyzed. Even though studies providing strong evidence for spatial structures in entire protist communities are still scarce (Porter and Hajibabaei 2018), our findings support recent molecular-based studies on eukaryote diversity and distribution patterns (e.g., Santoferrara et al. 2016), suggesting that ecologists should account for biogeographical patterns instead of assuming a ubiquitous dispersal for protists (van der Gast 2015).

In addition, once HTS-based studies capture a large proportion of the rare biosphere, the assumption that high population densities increase the potential for dispersal no longer holds. The rare biosphere could, therefore, present spatiotemporal distribution patterns more similar to that of macroorganisms, which could derive from dispersal limitation (Dunthorn et al. 2014). In fact, recent studies indicate that the rare biosphere exhibits biogeographic-scale patterns, which, in turn, may contribute to spatial distribution patterns of the entire community (Weisse 2014, Lynch and Neufeld 2015). It is noteworthy that although morphological studies fail to register a large part of rare species within a regional species pool, even in studies based only on microscope identification, large-scale spatial composition of rare ciliate species can be much clearer than for common species (Segovia et al. 2017).

Finally, metacommunity theory may also provide a foundation to explain the weak environmental filtering we found through the molecular approach. According to this theory, high dispersal rates may overcome the effects of local environmental conditions, such that poorly adapted species are able to persist in unfavorable environments because of dispersal surplus (Leibold et al. 2004). The stronger fine-scale spatial effect we found via the molecular-based approach leads us to infer that dispersal surplus may be captured in greater magnitude by this approach than the morphological approach. For example, when spatial factors are important, it is an indication that community variation is influenced by dispersal limitation or dispersal surplus (Ng et al. 2009). Following the premise that dispersal surplus potentially increases in importance with decreasing distance between sites (Heino et al. 2015), fine-scale spatial variables are more likely to evidence this mechanism, also known as mass effects (Brown et al. 2017). The spatial Moran's eigenvector maps, related to within-floodplain extent, were relevant only for the molecular approach, which indicates this approach's potentially greater ability to detect dispersal surplus. Further, as discussed above, the detection of homogenizing effects was greater in the molecular than morphological approach.

Implications of morphological- and molecular-based approaches

Differences in methodology and accuracy between morphological- and molecular-based approaches have important implications for characterizing microorganism assemblages. Morphological identification of ciliates presents several strengths and weaknesses. Not only is in-vivo processing of samples needed, but accurate identification to the lowest possible taxonomic level requires the use of impregnation techniques that allow for the observation of infraciliature and nuclear apparatus (Foissner et al. 1999). The use of these methods in biomonitoring is limited by high cost and a lack of people skilled in identification methods (Stoeck et al. 2014). Therefore, morphological identification based only on living organisms may lead to multiple different species, likely rare, being grouped into only 1 species. For instance, the morphospecies identified as *Paramecium*

caudatum, an easily recognizable and cosmopolitan ciliate, was first recorded in bromeliad tanks located near the banks of the Paraná River, but later impregnation techniques and molecular analyses identified it as *Paramecium multimicronucleatum* (Buosi et al. 2014). However, where species share functional traits (Vandewalle et al. 2010), morphological identification to the genus level may be preferable to species-level identity or molecular methods in order to avoid biasing the determination of species–environment relationships (Xu et al. 2011, Cabral et al. 2017).

For the molecular approach, the opportunities are countless but also extremely challenging. From a biomonitoring perspective, it is important to maintain the ecological relevance of OTUs without overinterpreting genetic polymorphism (Pawlowski et al. 2016). For example, species with different functional traits (e.g., dispersal modes, capacity of encystment) differ in their use of resources and their distributions, which directly affects the species-level fitness of the organisms (Violle et al. 2007). Therefore, where there is little or no information about how functional traits of ciliates might overlap among OTUs, it is suggested that biomonitoring protocols should consider environmental DNA metabarcoding in ecological assessment (Pawlowski et al. 2016). However, cryptic species still share similar functional traits that yield low niche differentiation between them, which may be determined by the same environmental filters (Stoks et al. 2005). Thus, although unraveling community composition into a high number of OTUs may strengthen the signal of spatial processes in community assembly, it is also important to account for molecular heterogeneities produced by polymorphism, which may result in empirical patterns with low actual contributions to ecological function.

Another challenge of using molecular data for bioassessment is that environmental DNA comprises both intra- and extracellular DNA, which can present issues around inferences drawn from molecular data. The presence of extracellular DNA can be very useful for biomonitoring and is widely used for the early detection of invasive and endangered species (Pawlowski et al. 2016). However, for bioassessment, the distinction between living, deceased, or inactive cells is necessary to predict the impacts of environmental changes on community composition (Ramírez et al. 2018). The presence of extracellular DNA retained in the sediment and transported in flowing waters creates undesired noise that may confound assessment of empirical patterns (Aylagas et al. 2016).

Study limitations

There are a few caveats in our study. From an ecological viewpoint, we recognize that dispersal among sites may involve a number of mechanisms and different propagule dispersal vectors (e.g., animal, physical) that are impossible for us to account for in this study. However, our sampling design involved only connected lakes and no sampling took place during flood periods, which minimized the importance of differences in site connectivity. In addition, we sampled only a few lakes (n = 5–11) in each floodplain, which could weaken our ability to detect the relationship between ciliate assemblage and the predictor variables in the variance partitioning analysis (Gilbert and Bennett 2010). Moreover, as is typical in studies of species–environment relationships, one can never measure all the potentially important environmental variables affecting organisms' distributions, which may lead to underestimating the effects of environmental filtering on community composition. Thus, it is important highlight that the use of spatial eigenfunction-based variables may lead to overestimation of the spatial effects on community composition (Gilbert and Bennett 2010). However, although some variation remained unexplained for both morphological- and molecular-based approaches (62 and 54%, respectively; Fig. 5A, B), a substantial amount was explained by environmental conditions and fine- and broad-scale spatial variables. Further, we used a combination of predictor variables, including physicochemical variables, nutritional resources (bacteria, flagellates, and phytoplankton), and potential predators (rotifers, cladocerans, and copepods), all recognized for exercising a strong influence on ciliate assemblage. Thus, it is unlikely that the relatively low explanatory power of predictor variables would be the consequence of under sampling and instead may reflect the importance of stochastic processes for community assembly (Nabout et al. 2009).

Another potential limitation of our study is related to the specific molecular analysis techniques we chose. Specifically, we used primers that amplified the V3 hyper-variable region of the 18S-rRNA locus. These general protistan primers have successfully been used in aquatic environments (Nolte et al. 2010), but the use of these V3 primers could also make it hard to compare with other aquatic and terrestrial studies of protists that used primers targeting other 18S-rRNA regions, such as the V9 region (Stoeck et al. 2010). Despite this methodological issue, we achieved our goal of discerning whether clear patterns in assemblage variation would be apparent between the morphological and methodological approaches. Further, species and OTUs are, in most cases, remarkably correlated (Pitsch et al. 2019).

Broader implications

Our results showed that site-by-site dissimilarity varied greatly across floodplain systems, independently of the approach used. Furthermore, the mechanisms shaping the composition of the ciliate assemblages showed striking differences between morphological- and molecular-based approaches, as expected. In general, morphologically-based composition of the ciliate assemblages was more strongly influenced by local environmental conditions (i.e., species sorting), whereas molecular-based composition was mainly influenced by broad-scale spatial processes (i.e., dispersal

limitation). In addition, dispersal surplus seems to be more pronounced in the molecular-based composition. This finding suggests that ecological research and biomonitoring activities need to find a counterbalance that uses the unique insights provided by each approach. On one hand, molecular OTUs detected by HTS include hidden diversity that can be fundamental for detecting biogeographical patterns. At the same time, morphological-based approaches may be useful bioindicators for monitoring water quality because they may more accurately reflect environmental gradients. Finally, long-term studies are needed to better design plans for managing and conserving aquatic biodiversity, and future studies would benefit from evaluating and comparing the temporal variation of assemblages based on morphological and molecular data, highlighting the opportunities and obstacles of each approach.

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