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Inland Saline Lakes as Hotspots of Specialized, Nonmarine Protist Diversity: The Case of Arcellinida (Amoebozoa)

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ABSTRACT

Inland saline lakes are dynamic environments with fluctuating salinity levels, sometimes reaching values higher than those of seawater. Their abiotic conditions are often extreme and more unpredictable than in marine systems, which could imply lower biotic pressures. This could have allowed nonmarine organisms to cross the salinity barrier and subsequently specialize and diversify there. Alternatively, inland saline lakes could host generalist species that are also found in other environments. In order to answer this question, we focused on a group of protists characterized by their narrow ecological tolerance, Arcellinida testate amoebae. We studied their diversity using a metabarcoding approach in different thalassic and athalassic saline systems from Spain and Chile. The majority of operational taxonomic units (OTUs) were exclusive to athalassohaline systems. Three clades included most diversity in athalassohaline systems, in addition to several specific colonization events. Inland saline systems have been colonized by freshwater species, which diversified there, turning these athalassohaline environments into hotspots of specialized Arcellinida biodiversity.

1 | Introduction

The salinity barrier is considered one of the most important frontiers dividing biodiversity and influencing the distribution of organisms (González-Miguéns, Soler-Zamora, Villar-Depablo, et al. 2022; Jamy et al. 2022; Snoeijs-Leijonmalm 2017). Crossing it opens the possibility for newcomer organisms to colonize new niches and diversify but requires substantial physiological adaptations to cope with the new osmotic pressures (Beadle 1969; Nakov et al. 2020; Oren 2001). These transitions are considered rare events in the evolutionary history of eukaryotes (Hutchinson 1975; Logares et al. 2009), although transition rates vary between clades (Jamy et al. 2022). Most studies on evolutionary transitions of the salinity barrier have focused on transitions between freshwater and marine environments (Lee and Bell 1999; Logares et al. 2009). However, fewer studies have documented such transitions within continents, that is, between freshwater/terrestrial habitats and inland saline systems (Deng et al. 2022; Stepanek and Patrick Kociolek 2019).

Athalassohaline ecosystems are saline but do not have any connection with the marine environment (Bayly 1967; Williams 2002). Athalassohaline lakes, such as salt flats or salares, are typically found in endorheic drainage basins of arid or semiarid environments (Hammer 1986; Williams 2002; Wurtsbaugh et al. 2017). Their salt concentration and ionic composition can vary greatly

[Correction added on 23 September 2025, after first online publication: An affiliation to Universidad Autónoma de Madrid has been added for Fernando Useros.]

between lakes and often fluctuate throughout the year. Salinity can range from near freshwater to values well above the 30–35 g/L of seawater, in some cases reaching values close to saturation (over 300 g/L) (Oren 2007; Safarpour et al. 2018; Williams 2002). For these reasons, the biodiversity of athalassohaline systems can be expected to differ deeply from the sea. Accordingly, early works already described important differences in species composition between athalassohaline and marine microbial communities (Bayly 1993; Hahn 2006; Timms 2020).

Many studies on athalassohaline water bodies have been fostered by the search for extremophilic life, thus focusing research interest on hypersaline lakes (Belmonte et al. 2012; Casamayor et al. 2013; Demergasso et al. 2004). While these studies are of crucial relevance to reveal which groups can withstand the limits of life, the key to understanding the phylogenetic pathways that allowed their colonization lies in less saline water bodies, which have been relatively overlooked. These less-extreme systems could represent stepping stones for ecological transitions across the salinity barrier and thus better models to understand these ecological transitions.

Large-scale studies focused on the distribution and diversity of a given taxon can help reveal the evolutionary history followed by the group to cross the salinity barrier (González-Miguéns, Soler-Zamora, Villar-Depablo, et al. 2022). For that purpose, environmental DNA (eDNA) sequencing provides a wealth of information for biodiversity and phylogenetic analysis (Adamowicz et al. 2019; West et al. 2021). Here, we will use Arcellinida testate amoeba as a model group to study this process. Arcellinida typically inhabit freshwater and terrestrial ecosystems (Fernández et al. 2015; Heger et al. 2011; Singer et al. 2018; Useros et al. 2024), but their presence in athalassohaline systems has been documented also: three new species were recently discovered in saline lakes in central Spain, where salinity frequently rose above that of seawater (Useros et al. 2023). Observational studies confirmed their presence in coastal, marine-influenced habitats but always at low salinities (Barnett et al. 2017; Gehrels et al. 2006; Golemansky 1991, 1998; van Hengstum et al. 2008; Whittle et al. 2019). However, these studies were based only on morphological identifications. Given the high level of cryptic diversity among species with different ecologies (Bickford et al. 2007; Oliverio et al. 2014; Singer et al. 2018), it remains unclear whether these specimens genuinely belonged to these marine systems or if they were freshwater species that drifted there accidentally.

The taxonomy and ecology of Arcellinida is relatively well known: Arcellinida is divided into three suborders: Phryganellina, with only 46 described species (Bobrov and Mazei 2017; Dumack et al. 2020); Organoconcha, with about 15 species; and Glutinococha, the most conspicuous and well-studied, comprising the great majority of the described Arcellinida species (> 680) and subdivided into at least 6 infraorders (González-Miguéns, Todorov, Blandenier, et al. 2022; Lahr et al. 2019; Useros et al. 2024). Within those infraorders, Excentrostoma and Sphaerothecina are the most diverse and common in aquatic habitats (González-Miguéns et al. 2023; Meisterfeld 2002). Arcellinida are generally considered ecological specialists; many species have narrow niches and are highly sensitive to environmental perturbations (González-Miguéns et al. 2024; Singer et al. 2018), hence their use in bioindication (Creevy et al. 2018; Delaine et al. 2016; Kosakyan and Lara 2019; Roe and Patterson 2014) and paleoecology (Mitchell et al. 2008). Because of their sensitivity to environmental conditions and their relatively well-established systematics, Arcellinida can be considered an excellent model group to study ecological transitions through the salinity barrier (Useros et al. 2023).

Due to their narrow ecological niches, a possible evolutionary scenario ("specialist hypothesis") is that some Arcellinida species have adapted to saline systems and diversified there. In that case, communities from saline systems would differ from those in other environments, and specific clades, formed by halotolerant/halophilic species should appear in phylogenetic trees. Such a scenario has been described in other examples of transitions across the salinity barrier like in killifishes (Whitehead 2010) and Cyphoderiidae protists (González-Miguéns, Soler-Zamora, Villar-Depablo, et al. 2022). Alternatively, saline systems could host generalist species whose wide ecological tolerance would allow them to live in saline systems ("generalist hypothesis"). If this was the case, such generalist species would be found also in non-saline systems and would not form specific clades. We will test here both hypotheses, based on a sampling design including inland saline, marine-influenced, and freshwater samples from both hemispheres, in order to evaluate how far evolutionary scenarios are valid for both marine and continental ecosystems.

2 | Methods

2.1 | Sampling Protocol and Study Area

We took 3–4 sediment samples at different points in each site, separated by at least 10 m. We sampled 5 mL of the upper 5 mm of sediment with a clean disposable plastic Pasteur pipette on the edge of water bodies, preferably next to algal mats or aquatic vegetation, and placed them in a Falcon tube with 5 mL of water from the site. 1 mL of sediment was fixed in the nucleic acids preservation buffer LifeGuard (Qiagen) (1:1 ratio) for later eDNA extraction, the rest was stored in the Falcon tubes at 4°C for subsequent microscopic observations and single cell isolation. LifeGuard has been shown to efficiently extract nucleic acids, providing high yield and quality in hypersaline, marine, and freshwater sediments (Keneally et al. 2024). In Chile salinity, temperature and other environmental parameters were measured in situ with a multiparameter probe (HI9829, Hanna Instruments) and pH with a pH tester (HI98127). In Spain, salinity was measured with a water refractometer (HI 96822) and pH and temperature with the pH tester (HI98127). All measured parameters and their respective values are available in Table S1. At each site, we also took terrestrial samples, which consisted of bryophyte cushions or leaf litter, to compare the diversity found there and in the aquatic habitat.

Our study area included three main regions, namely (1) central Spain; (2) central Chile; and (3) northern Chile (Figure 1, Table S1). The coordinates, dates, and environmental characteristics of all sampling sites can be found in Table S1.

2.1.1 | Central Spain

The samples from central Spain are from two areas, both of them under a Mediterranean-continental climate. The first site, Salobral

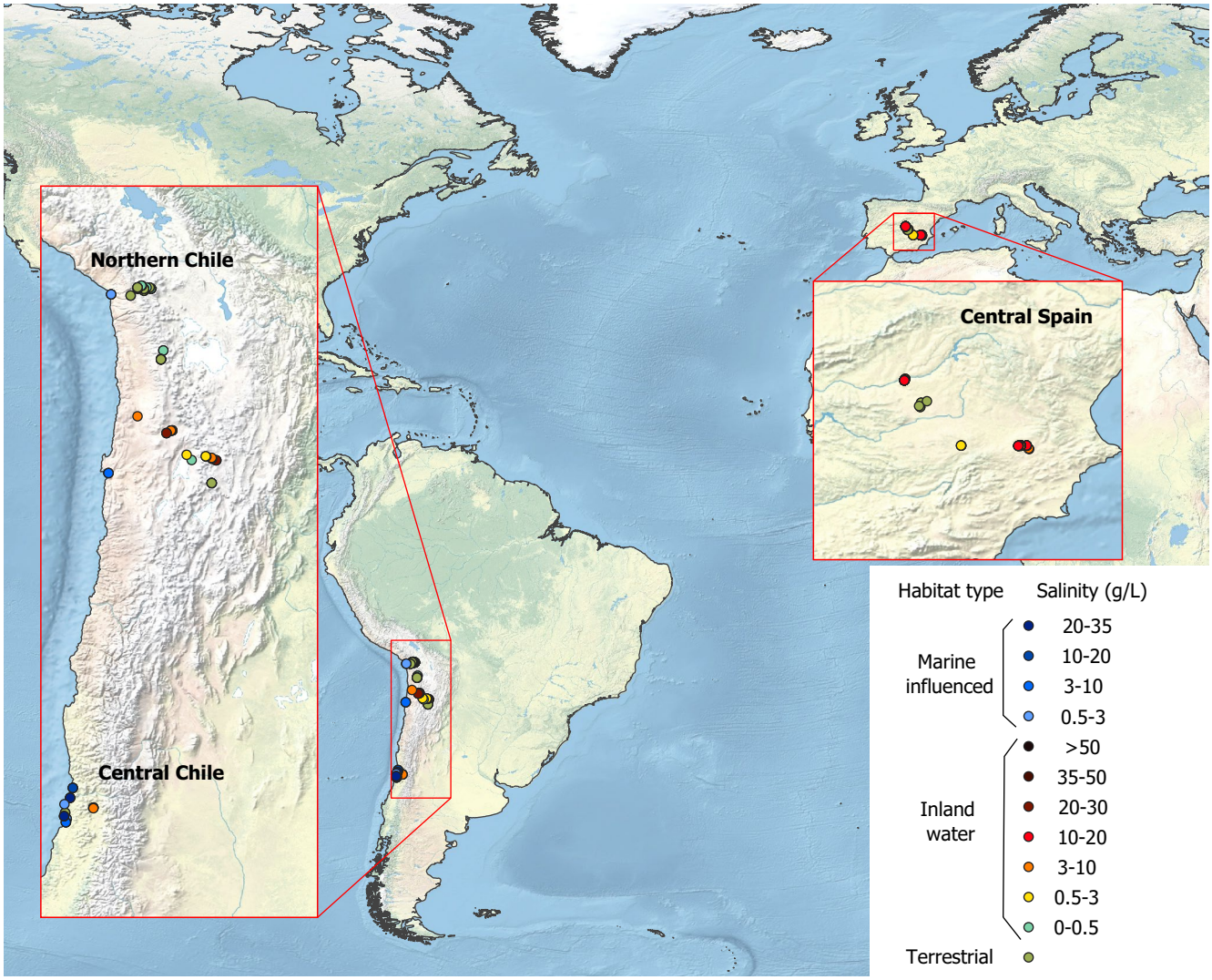

FIGURE 1 | Sampling sites, colored by type of habitat and salinity level.

de Ocaña, is a saline wetland located on a gypsum-rich soil formed by several streams and temporary lakes. A literature survey showed that the main ions present consist of SO_4^{2-}, Ca^{2+}, and Na^+ (Menéndez Pidal et al. 2021). A second site included the athalassohaline lakes Horna, Petrola, and Corral Rubio, whose ionic composition is mainly Mg^{2+}, Na^+, $Cl,^-$ and SO_4^{2-} (Valiente et al. 2017).

2.1.2 | Central Chile

Inland samples were collected from the Batuco Lagoon in Santiago de Chile, Metropolitan Region, an area under a Mediterranean-semiarid climate (Del Campo et al. 2005). We could not find data on the ionic composition of this lagoon. We also sampled several coastal lagoons and estuaries in the Valparaiso region to compare the diversity and patterns found in athalassic and thalassic saline systems.

2.1.3 | Northern Chile

We sampled athalassohaline and freshwater sites within the Altiplano Plateau. This arid region is home to some important high-altitude (2500–4500 m a.s.l.) wetlands and salt lakes, which include some of the largest salt flats in the world (Marazuela et al. 2019; Paquis et al. 2023). The formation of the Altiplano Plateau began approximately 25–10 million years ago during the late Oligocene to early Miocene, as a result of tectonic uplift caused by the subduction of the Nazca plate beneath the South American plate (Garzione et al. 2008). This geological process contributed to creating rain shadows that reduced moisture from the Pacific and vast endorheic basins in which salts accumulated over time, eventually forming salt flats, also known as salares (Alonso et al. 1991; Alonso and Rojas 2020; Houston and Hartley 2003). Salt composition usually includes K^+, Mg^{2+}, Na^+, SO_4^{2-}, and Cl^- (Marazuela et al. 2019; Munk et al. 2016). We also sampled a coastal lagoon in Antofagasta and an estuary in Arica.

2.2 | Single Cell Barcoding

A few milliliters of sample were transferred to a Petri dish to be observed under an inverted microscope Labomed TCM400, up to 400× magnification. Observed living individuals were isolated with a stirred Pasteur pipette, transferred to a drop

of sterile water and rinsed, and then further rinsed in another drop before being stored individually in Eppendorf tubes with 100 μL of guanidine thiocyanate-based nucleic acids extraction buffer (Chomczynski and Sacchi 1987). Single cell DNA extractions were performed based on the protocol by Duckert et al. (2018). The tubes were incubated for 25 min at 65°C, then we added 60 μL of isopropanol at −80°C, vortexed, and left overnight at 4°C. Then we centrifuged for 20 min at 15,000 rpm, removed the supernatant and did another two washing steps, with 70% and absolute ethanol respectively, and 5 min centrifugations. The residual ethanol was evaporated for 1 h under a fume hood. Then 5 μL of miliQ water were added to resuspend nucleic acids.

We amplified a portion of the cytochrome oxidase subunit I (COI) using a two-step seminested polymerase chain reaction (PCR) as described in González-Miguéns et al. (2023), which can be seen in detail in dx.doi.org/10.17504/protocols.io.yxmvm2389g3p/v1. In summary: A first amplification was performed with the primers LCO 1490 (5′ GGTCAACAAATCATAAAGATATTGG 3′) and HCO 2198 (5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) (Folmer et al. 1994). We diluted these PCR products 1:20 and performed the second PCR using the Arcellinida-specific primer ArCOIR (5′ CCACYNGAATGWGCTARAATACC 3′) (González-Miguéns, Soler-Zamora, Useros, et al. 2022) paired with LCO 1490 and ArCOIF (5′ GGTATTYTAGCWCATTCNRGTGG 3′) paired with HCO 2198. We used MyTaq Red DNA polymerase Mix (Bioline) for all PCRs. Final products with bands of expected size (406 bp) were purified by band excision and stored at −20°C. Products were sequenced in both forward and reverse directions using Sanger dideoxy-technology by Secugen S.L (Madrid, Spain).

2.3 | eDNA Extraction, Amplification, and Sequencing

eDNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's protocol. In short, the process involved the disruption of soil particles using bead beating, followed by a series of purification steps, including the binding of DNA to a silica membrane and washing steps to remove contaminants. The extracted DNA was then eluted in a low-salt buffer and stored at −20°C or −80°C for downstream applications. This kit is commonly used to extract eDNA from soil and sediment samples, including saline samples (Xie et al. 2018). The PCR amplification protocol was the same as before, but with 1 μL of BSA (initial concentration: 10 mg/mL) in the first step (González-Miguéns et al. 2023) and using indexed ArCOIR and LCO 1490 primers in the second PCR (list of indexed primers used provided in Supporting Information S1). Final products with bands of expected size (422 bp) were purified by band excision. DNA concentration was measured using a Qubit 3 fluorometer and a dsDNA high sensitivity assay kit (ThermoFisher). We pooled the samples in equimolar amounts so that the final concentrations of each sample were normalized. Library preparation and sequencing of the pool was performed by the company "Fundación Parque Científico de Madrid" (Madrid, Spain), with Illumina MiSeq v2, generating paired-end 250 bp reads.

2.4 | Data Curation; Avoiding the Effect of Tag Jumping

Illumina reads were curated following the pipeline in González-Miguéns et al. (2023) (scripts provided in Supporting Information S1). Trimming of primers and demultiplexing was done with cutadapt v2.8 following the pipeline "Cutadapt_pipeline.bash." The resulting reads per sample were analyzed with the dada2 R package (Callahan et al. 2016) following the tutorial in https://benjjneb.github.io/dada2/tutorial_1_8.html, with minor modifications, from González-Miguéns, Soler-Zamora, Villar-Depablo, et al. (2022) (dada2 outputs available in Supporting Information S2). We used VSEARCH v 2.14 (Rognes et al. 2016) for taxonomic assignment using a curated database based on the one from González-Miguéns et al. (2023), which included sequences from GenBank of Amoebozoa, Excavata, SAR, Opisthokonta, and Archaeplastida. We filtered out a priori those amplicon sequence variants (ASVs) that had an identity value for Arcellinida lower than 80% (1507 ASVs remained out of 11,799); this threshold has been determined empirically (González-Miguéns et al. 2023). To remove misassigned sequences due to errors and tag-jumping, which can substantially distort metabarcoding datasets creating false positives (Rodriguez-Martinez et al. 2023; Schnell et al. 2015), we removed observations whose occurrence was below certain thresholds. We determined those thresholds by observing the reads with primer combinations that were not used (which we considered during the bioinformatics analysis as blank samples), which can be considered as known errors. Highly abundant ASVs have a higher probability of generating tag-jumping biases (Rodriguez-Martinez et al. 2023). Reads associated to the wrong sample due to tag jumping may then be numerous in certain cases, but they should represent a low proportion of the total number of reads of a given ASV. Furthermore, some reads may have been amplified less efficiently, for instance because of mismatches in the priming regions or because the organisms are simply rarer, resulting in a low number of reads, but in that case they should represent a higher proportion of the total number of reads of that ASV. In sum, we considered both the number of reads of an ASV present in blank samples and the proportion those reads represented with respect to the total number of reads of that ASV across all the samples.

We used the melt function from the R package reshape v0.8.9 (Wickham 2007) on the dada2 matrix output to generate a dataframe in which each row represents an observation, that is, the number of reads of one ASV in one sample. That way, we could add extra columns with information about the sample (coordinates, physicochemical parameters, if it was a blank sample, etc.) and the ASV (total number of reads of that ASV, taxonomy...) for further analysis. Blank samples contained six exclusive ASVs (only present in blank samples), most probably the result of sequencing errors beyond tag jumping. They all had less than 10 reads, for this reason we removed observations with 10 or less reads from our dataset as possible errors (1304 observations and 808 ASVs remained). Then, the most abundant observation in one of those blank samples had 1696 reads, which represented 0.4% of the total number of reads of that particular ASV. On the other hand, the observation with the highest proportion of reads with respect to the total number of reads of an ASV had a 5.95%, with 55 reads. We considered both of those maximum values, adding 10% to have a security margin, as cutoff. Thus, if

the number of reads of an ASV in a sample was lower than 1866 and those represented less than 6.54% of the total proportion of reads of that ASV, we removed those observations as potential tag-jumping artifacts. 823 observations remained after this filter (complete table with ASV and site information available in Supporting Information S3).

This dual threshold would produce fewer false negatives than using only one of these values. Still, some abundant ASVs could be naturally less abundant in certain samples and filtered out, thus generating false negatives. We chose conservative values willingly, knowing that false positives in our case would harm much more the interpretation of our results than false negatives. In practice, it is impossible to determine objectively a method that can rule both of them out. The trade-off between the relevance of retrieving false positives or negatives depends on the objective of each study.

2.5 | Clustering Into OTUs and Phylogenetic Analysis

We aligned the sequences using the auto MAFFT algorithm (Katoh et al. 2002), as implemented in Geneious Prime (v. 2019.0.4). We included in this alignment also barcoded sequences, sequences from Genbank as well as from another metabarcoding study by González-Miguéns et al. (2024) that used the same protocol. This alignment was manually edited using Aliview (v. 1.27) (Larsson 2014) (alignment available in Supporting Information S4). Then, we generated a phylogenetic tree with Maximum likelihood analysis on the online version of IQ-TREE (Nguyen et al. 2015; Trifinopoulos et al. 2016), allowing it to search for the best substitution model with free rate heterogeneity and performing 10,000 non-parametric bootstraps to assess node supports (tree file available in Supporting Information S4). We generated a patristic distance matrix in Geneious Prime. We used the R package DECIPHER v2.22 (Wright 2016) and that distance matrix to cluster the sequences into operational taxonomic units (OTUs). We used a molecular pairwise distance of 0.03 as cutoff ("barcoding gap") and the "complete" method, meaning that there is a maximum distance of 0.03 between all the ASVs of an OTU. Our rationale behind that choice is that 0.03 is the distance that separated the sibling species *Arcella salobris* and *Arcella uspiensis*, the two described species with the lowest pairwise distance in the COI Arcellinida database. Both species are known to differ strongly in their salinity tolerance, geographical distribution, and slightly in their morphology, which justifies altogether that they can be considered as separate taxonomic entities (Useros et al. 2023). Therefore, by using this threshold (barcoding gap), we aim at obtaining OTUs that are most akin to species under a conservative species delimitation approach (Supporting Information S5 indicates which ASVs or barcoding sequences were clustered into each OTU).

When several ASVs constituted a single OTU, we considered the ASV with most reads as the reference sequence for the subsequent alignment to facilitate the graphical representation of the results. We aligned and generated a phylogenetic tree for the OTUs as before. We used the R package ggtree v3.2 (Yu 2020) to graphically represent the tree with ecological and taxonomic information related to each OTU.

Finally, we used the R package pegas v1.2 (Paradis 2010) to generate haplotype networks of the OTUs that had several haplotypes and that were found in different habitats or regions to see if there was a structure between the haplotype network and those variables.

2.6 | Diversity Analysis

We classified the samples into different habitat categories: sediment from marine-influenced water bodies, from inland water bodies or terrestrial samples. The sediment samples were further classified according to salinity ranges (Beadle 1943; Hammer et al. 1983). We first determined if there was a difference in the proportion of samples with and without Arcellinida among the different habitats with the function ggbetweenstats from the R package ggstatsplot (Patil 2021). We also compared samples from marine influenced (thalassic) versus athalassohaline water bodies that fell within the range of the coastal samples we had (0.5–28 g/L).

A prerequisite to compare diversity between samples is that the richness is not correlated with the number of reads per sample, as that would indicate a bias introduced by sequencing depth (González-Miguéns et al. 2024). First, we adjusted a linear model between OTU diversity and sequencing depth (number of reads) with the lm function of the R package stats v4.3.2 (R Core Team 2022), which gave us a significant relationship ($p = 2 \times 10^{-16}$). Then, we compared the sequencing depth in the different habitats. We used the shapiro_test and levene_test functions of the R package rstatix (Kassambara 2021) to verify the normality and homoscedasticity of the data, respectively. Then, we used the function ggbetweenstats, indicating if the data were normal and homoscedastic according to the previous tests, to compare the sequencing depth in the different habitats. These relationships were significant ($p = 0.02$), which implies that richness between samples could not be directly compared in terms of number of OTUs. Therefore, in order to compare the diversity of the different habitats surveyed removing the sequencing depth bias, we used the residuals of the linear model between OTU diversity and sequencing depth, which are the residual variances in richness not explained by sequencing depth (Hiiesalu et al. 2014; Lentendu et al. 2023). Therefore, if the residual is positive the sample has more richness than what the model would predict according to its sequencing depth. This removes the sequencing depth bias avoiding the application of a rarefaction step, considered as detrimental in microbiome analysis because it requires the omission of available valid data (McMurdie and Holmes 2014). We used the same functions as before, plus a pairwise *t* test with the Holm *p* adjustment method, to compare the residuals of the samples from different habitats.

Then, we searched for a correlation between salinity and diversity in inland water bodies. We took into account only samples with salinities below 37 g/L, because we did not observe Arcellinida beyond 30 g/L. We checked, as before, if there was a correlation between sequencing depth and salinity; since there was not ($p = 0.54$) we did not need to correct for sequencing depth and could use OTUs numbers directly as variables to compare diversity. Since the Shapiro–Wilk test indicated that the distribution was not normal, we used the Spearman method in the function cor.test.

3 | Results

3.1 | Salinity Levels of the Sampling Sites

The salinity in our marine-influenced samples ranged from 0.52 to 27.9 g/L and in inland aquatic samples from 0 to 50.3 g/L, although most of them are below 15 g/L. There was a high variability in salinity levels between and sometimes also within localities. In some systems, there were large salinity differences between the sampling points, for example, from 3.2 to 40.3 g/L in Salar Grande (Chile) or from 2.7 to 50.3 g/L in Lake Pétrola (Spain) (all sampling sites information in Supporting Information S1).

3.2 | Presence and Biodiversity of Arcellinida in Different Environments

We successfully barcoded 24 single-cell individuals, which corresponded to 11 different OTUs (Table 1). Five of these OTUs were not recovered in the eDNA survey. Figure 2 shows photographs of some of the barcoded specimens (more photos in Supporting Information S1).

We obtained a total of 808 Arcellinida ASVs, which were clustered into 323 OTUs delimited using the 3% barcoding gap as previously set. The proportion of samples with or without Arcellinida from the different environmental categories only was significantly higher in freshwater samples versus marine-influenced (0.5–3 g/L salinity) and athalassohaline samples over 35 g/L; the rest had no significant differences (Figure 3a). Nevertheless, this proportion was significantly lower ($p = 1.2 \times 10^{-3}$) in marine-influenced environments than in athalassohaline environments with salinity levels within the same range (0.5–28 g/L, the range we have of marine-influenced samples) (Figure 3b). Also, the highest salinity where we found Arcellinida in marine-influenced water bodies was 2.86 g/L, whereas in athalassohaline water bodies it was 28.3 g/L.

We did not observe significant differences in Arcellinida OTU diversity after correcting for the sequencing depth of the samples ($p = 0.21$), probably because there was a high variance between samples from the same category (Figure 4b). However, taking only inland aquatic samples, we observed a significant decrease in OTU richness as salinity increases (adjusted linear model's $p = 0.017$ and adjusted $R^2 = 0.078$, Spearman's correlation $p = 10^{-4}$ and Spearman's rho $= -0.48$) (Figure 4c).

TABLE 1 | Information about the barcoded specimens. Optic microscope photographs of some of the specimens can be found in Supporting Information S1.

Specimens' code	Identification	Infraorder	Genbank code	Corresponding OTU
A24_4	*Centropyxis* sp.	Excentrostoma	PP434329	OTU1
A24_17, A24_1, A21_4	*Centropyxis* sp.	Excentrostoma	PP434330	OTU1
A55_15, A55_17, A55_14, A4_4, A49_20	*Centropyxis* sp.	Excentrostoma	PP434331	OTU1
A21_21	*Centropyxis* sp.	Excentrostoma	PP434332	OTU5
A49_9	*Centropyxis* sp.	Excentrostoma	PP434333	OTU60
A50_14	*Centropyxis* sp.	Excentrostoma	PP434334	OTU60
A58_69	*Centropyxis* sp.	Excentrostoma	PP434335	OTU60
A5_4	*Centropyxis* cf. *discoides*	Excentrostoma	PP434336	(Not in metabarcoding)
A28_4	*Centropyxis* cf. *halophila*	Excentrostoma	PP434337	OTU58
A21_10	*Centropyxis* cf. *halophila*	Excentrostoma	PP434338	OTU58
A56_4	*Centropyxis* sp.	Excentrostoma	PP434339	OTU59
A56_11	*Centropyxis* sp.	Excentrostoma	PP434340	OTU59
A58_10	*Centropyxis* sp.	Excentrostoma	PP434341	OTU59
A50_7	*Difflugia* sp.	Longithecina	PP434342	(Not in metabarcoding)
A31_29	*Cylindrifflugia* sp.	Cylindrothecina	PP434343	OTU13
A56_17	*Phryganella* sp.	Phryganellina	PP434344	(Not in metabarcoding)
A56_c1	*Arcella* sp.	Sphaerotecina	PP434345	(Not in metabarcoding)
A56_c3	*Galeripora* sp.	Sphaerotecina	PP434346	(Found in González-Miguéns et al. (2024), metabarcoding data)

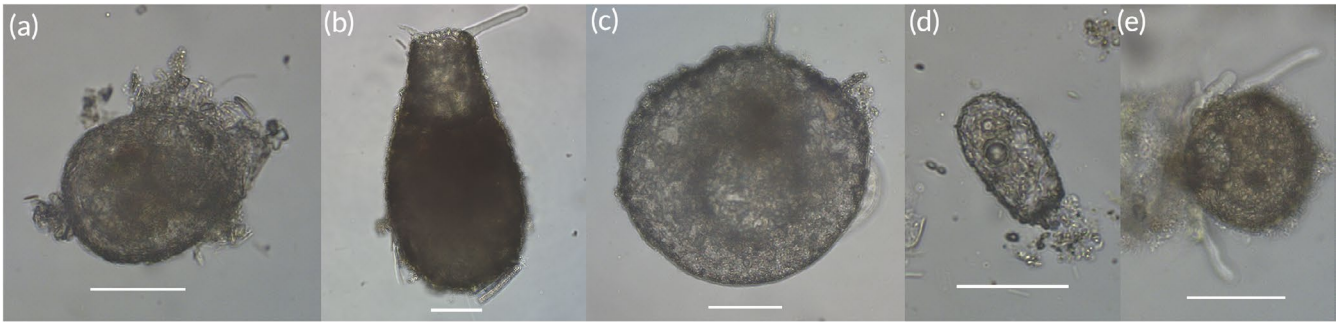

FIGURE 2 | Optical microscopy photographs of different barcoded specimens from three infraorders. White bar represents 50 μm. (a) *Centropyxis* sp., barcoded individual A24_17, in OTU1. (b) *Difflugia* sp., barcoded individual A50_17. (c) *Centropyxis* cf. *discoides*, barcoded individual A5_4. (d) *Cylindrifflugia* sp., barcoded individual A31_29, in OTU13. (e) *Centropyxis* sp. barcoded individual A56_11, in OTU59 (more photos in Supporting Information S1).

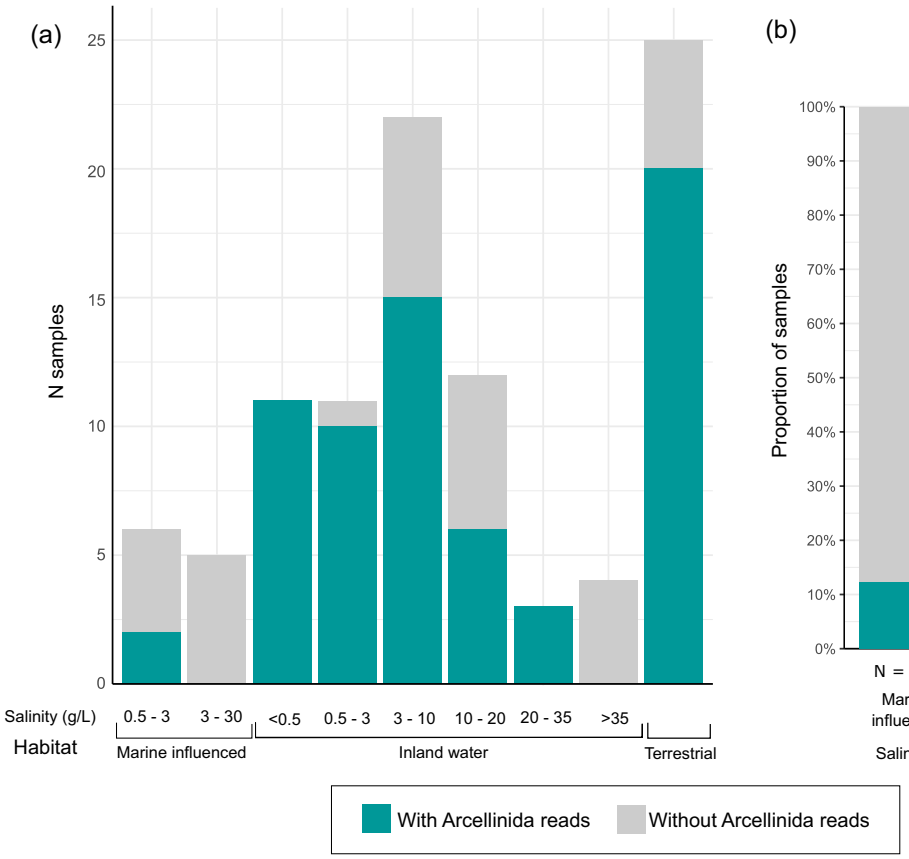

FIGURE 3 | (a) Number of samples with or without Arcellinida sequences from each habitat category. (b) Comparison of the proportion of samples with or without Arcellinida sequences from marine-influenced environments, with salinity ranging from 0.5 to 28 g/L, and from athalassohaline environments with salinity levels within that same range.

Furthermore, we did not find significant differences in the ASV/OTU ratio in samples from different habitats ($p = 0.36$), nor when we compared freshwater and the whole set of athalassohaline samples ($p = 0.46$).

The vast majority of OTUs, 273 out of 322 (84.8%), were found in only one sample and only 15 (4.7%) were found in three or more samples (Supporting Information S6). These results remain comparable even when grouping samples by site: 283/322 (87.9%) were found in only 1 site and only 11 (3.4%) in 3 or more. Consequently, the number of OTUs present in different environments is low (27 OTUs), as well as the number of shared OTUs between regions (10 OTUs); notably, 5 of these OTUs were found in both Spain and Chile (Supporting Information S4).

3.3 | Phylogenetic Placement and Ecological Transitions

The great majority of the OTUs found in the metabarcoding belonged to infraorder Excentrostoma (289 out of 322 OTUs), followed distantly by Phryganellina (13 OTUs) and Sphaerotecina

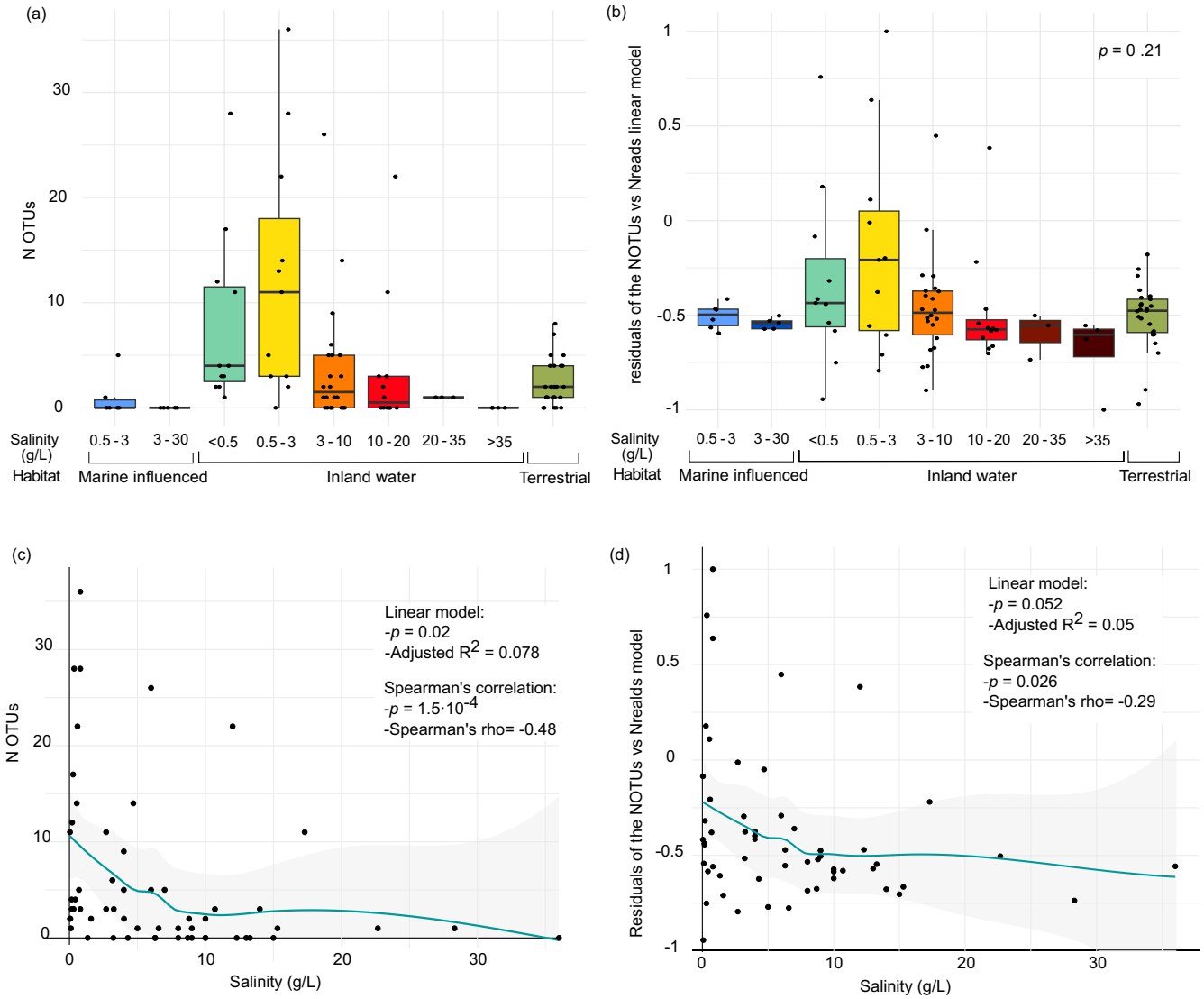

FIGURE 4 | Diversity of Arcellinida in samples from each habitat category, considered as (a) raw number of OTUs and as (b) residuals of the adjusted linear model between number of OTUs and sequencing depth of the sample. (c) Correlation between number of OTUs (significant correlation) and salinity in non-coastal aquatic environments. (d) Correlation between residuals of the number of OTUs versus number of reads model and salinity in non-coastal aquatic environments (significant Spearman's correlation, marginally significant linear model correlation).

(11 OTUs). We did not recover any Longithecina OTUs from the environmental sequences, despite isolating and successfully barcoding one specimen of that infraorder from one of the samples.

We found 220 OTUs/species in athalassohaline environments (113 in Chile, 112 in Spain, 5 of them were in both countries), 129 of them at salinities above 3 g/L. Very few of those were also found in freshwater environments (4 out of 32 in Chile and 3 out of 102 in Spain). On the other hand, there were only 17 OTUs found in marine-influenced environments, which were observed always below 3 g/L. Five of these coastal OTUs were also found in freshwater samples, and five were also found in athalassohaline environments too (Figure 5), some at salinities up to 17 g/L.

The OTUs retrieved from saline systems occupy a variety of positions in the Arcellinida tree, which suggests different independent adaptations to salinity (Figure 5). However, some sequences found exclusively in saline systems group together, forming three distinct clades within Excentrostoma; clades *Hal1* and *Hal2* appear predominantly in hypohaline samples, and clade *Hal3* in hypohaline and subhaline (Figure 6). All these three clades, as well as the terrestrial clade *Terr*, are nested within freshwater groups.

Most of the OTUs from the *Hal1* and *Hal2* were found in Central Spain, although some of them were found in Chile; there was no obvious pattern indicating single colonization events from one continent to the other (Figure S7). Five OTUs were found in both Spain and Chile, and only OTU56 was found in the three sampled regions.

3.4 | Haplotype Network Structure

The majority of the 322 OTUs retrieved in the metabarcoding had only 1 (240 OTUs, 74.5%) or 2 ASVs (49 OTUs, 15.2%).

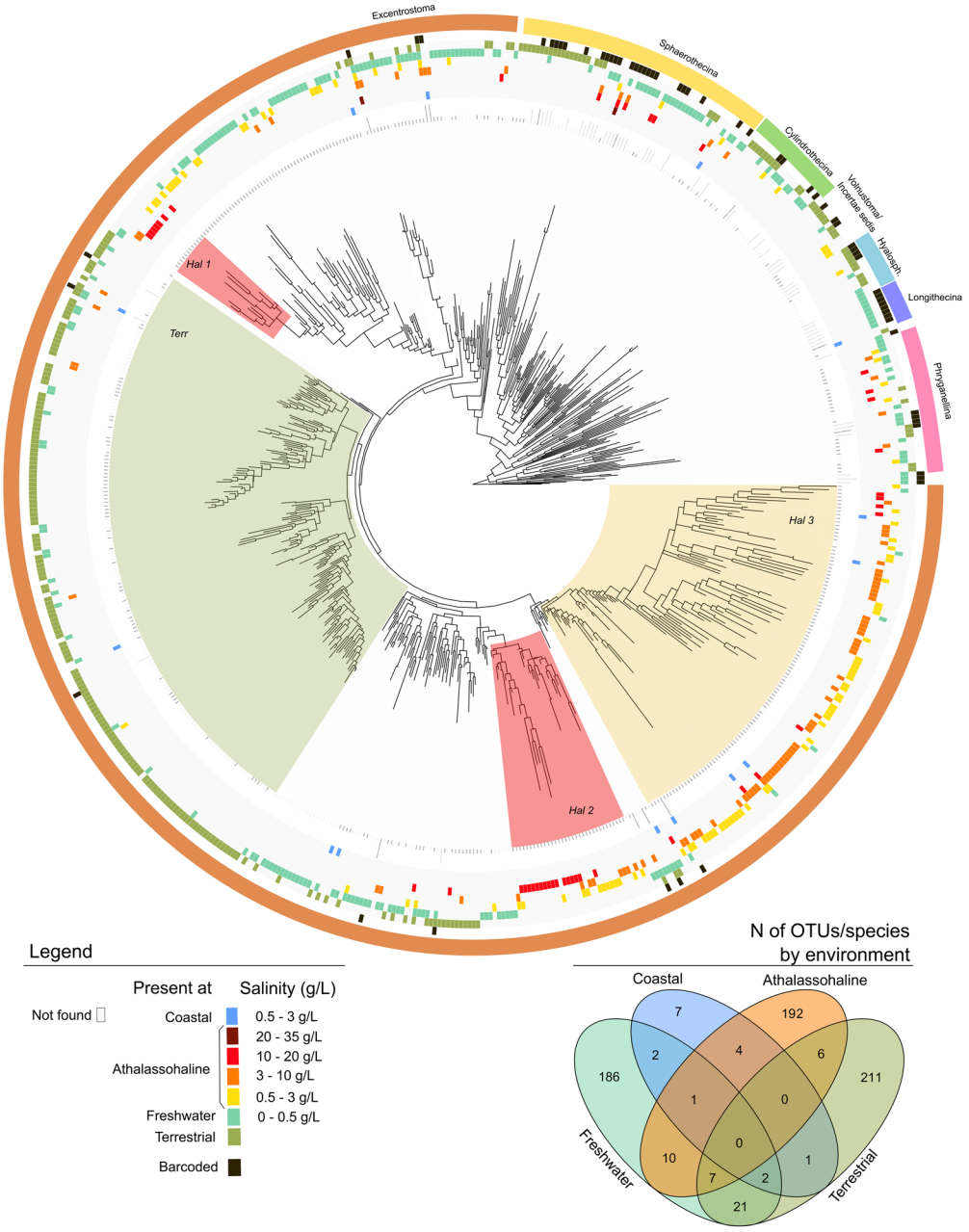

FIGURE 5 | Phylogenetic tree including sequences from barcoded specimens and metabarcoding data from this study and from González-Miguéns et al. (2024). Colored squares indicate in which habitats they were found. The shaded areas indicate clades that seem to have a tendency to live in a similar habitat type (terrestrial in clade *Terr*, athalassohaline environments in *Hal1*, *Hal2* and, with lower salinity levels, *Hal3*). The names of species and OTUs that were not found in our samples appear in gray. Outer color rings indicate the infraorder to which it belongs. The Venn diagram at the bottom summarizes the number of OTUs/species reported in each habitat type.

Only 7 OTUs (2.2%) had 10 or more ASVs, the maximum being OTU1 with 80 ASVs. The haplotype networks show that most of the OTU diversity tends to be located in one of the categories (habitat or region), without a clear pattern between the category of the ASV and its position in the network. For example, most of the ASVs of OTU1 were found in subhaline systems; the 11 ASVs found in mesohaline samples are dispersed in the network without forming any kind of cluster, and the same occurs with the 10 ASVs found in freshwater (Figure 6).

4 | Discussion

4.1 | Some Considerations on Arcellinida Biodiversity

The diversity of Arcellinida in athalassohaline water bodies was surprisingly high considering the dynamic conditions of these environments (Demergasso et al. 2004; Oren 2007; Safarpour et al. 2018) and how different they are from the

"typical" freshwater and soil Arcellinida habitats (González-Miguéns et al. 2023; Singer et al. 2018). The majority of the OTUs recovered, both in saline and non-saline environments, belonged to the infraorder Excentrostoma, which was also the infraorder predominantly recovered by González-Miguéns et al. (2023) in their seminal work on Arcellinida metabarcoding. These results are in line with earlier reports based on microscopical observations that also showed a dominance of this infraorder in coastal subhaline systems (Charman et al. 2002; Gehrels et al. 2006). Excentrostoma seem therefore to be the most diverse and abundant infraorder in all the sampled environments.

However, several of the sequences obtained by barcoding single cells, most of them of infraorders other than Excentrostoma, were not recovered in the metabarcoding even though it included the same samples. This suggests that part of the diversity still remains hidden, possibly due to primer or DNA extraction biases (Useros et al. 2024).

4.2 | Arcellinida in Saline Systems: Specialists or Generalists?

Salinity was negatively correlated with diversity in athalassohaline environments (Figure 4c), in line with what has been observed in many other groups of organisms, like bacteria, plants, diatoms, ciliates or arthropods (Demergasso et al. 2004; Hammer 1986; Pedrós-Alió et al. 2000; Rodriguez-Valera et al. 1985). Nevertheless, most of Arcellinida diversity found in these saline systems is endemic. Only 8.2% of the 220 OTUs found in athalassohaline samples appeared also in freshwater (and only 4.7% of those found above 3 g/L). These results show that athalassohaline water bodies host diverse Arcellinida communities that are distinct from other environments and that cannot be considered as a subset of other more stable ecosystems; in other terms, they fit at best with the "specialists hypothesis."

In contrast, 29.4% of the 17 OTUs found in marine-influenced samples were also found in freshwater, and also 29.4% in athalassohaline environments. Furthermore, none were found beyond 3 g/L in marine influenced environments, a result in line with morphology-based studies in coastal salt marshes (Barnett et al. 2017; Charman et al. 2002; Gehrels et al. 2006). This suggests that Arcellinida found in marine influenced environments tend to adhere more to the "generalists hypothesis."

4.3 | Inherited Adaptation to Athalassohaline Systems

We found several OTUs exclusively in athalassohaline systems that branched close to freshwater species, suggesting recent transitions across the salinity barrier, as in the case of *Arcella salobris* and *A. euryhalina* (Useros et al. 2023). There were also a few OTUs found in a wide range of salinities and environments. These are probably colonist species that have a wide ecological tolerance, which suggests a broad ecological spectrum. A good example is to be found in OTU1 (=*Centropyxis* sp.), which has been found under all salinity levels and whose haplotype network has no structuration by habitat (Figure 6), indicating that it is generalist and euryhaline. However, the phylogenetic placement of the majority of athalassohaline OTUs corroborates the hypothesis that Arcellinida found in these environments tend to be specialists (Figure 5). Indeed, our phylogenetic analyses revealed three clades of Excentrostoma found almost exclusively in athalassohaline environments. This suggests that there was a colonization of athalassohaline systems that was followed by diversification within this environment, a pattern commonly observed after ecological transitions and successful colonization events (Betancur et al. 2012; González-Miguéns, Soler-Zamora, Useros, et al. 2022; Kolbe et al. 2011; Whitehead 2010). In all cases, athalassohaline clades are nested within freshwater groups, which suggests that the ancestors lived originally in freshwater ecosystems.

The situation appears to be different in marine-influenced systems. Here, the sequences retrieved did not branch together or form any marine specialized group. This, coupled with the fact that we did not find any Arcellinida OTU in coastal systems above 3 g/L salinity and that a higher proportion of the OTUs found there were also present in other environments, suggests that we still did not find Arcellinida clades genuinely specialized in this marine-influenced environment. This apparent absence may seem counterintuitive, given the fact that athalassohaline Arcellinida species are capable of adapting to changing salinity levels (Useros et al. 2023). It has been suggested that biotic pressures, like competition with marine organisms already adapted to high salinity levels, are responsible for this absence (Useros et al. 2023). These biotic pressures would be lower in athalassohaline systems due to their island-like nature, which would have prevented their colonization by (marine) competitors and predators (Browne 1981; MacDonald et al. 2018). This allowed freshwater organisms like Arcellinida undergoing the process of adaptation required to cross the salinity barrier and colonize them. There are other examples of organisms that are found in athalassohaline or hypersaline systems but not in the sea, like Anostraca (Eng et al. 1990; Mura 1999), the green algae *Dunaliella* (Assunção et al. 2012; González et al. 2019) and some genera of Rotifera, Cladocera and Copepoda (Zsuga et al. 2021). However, there are also clades of marine Rotifera, Cladocera, Copepoda and green algae (Umaña-Villalobos et al. 2023). Arcellinida is an interesting case of a mainly freshwater order of protists with a few clades that have been able to cross the salinity barrier in athalassohaline environments, but none in marine-derived systems.

Athalassohaline environments are, therefore, hotspots for endemic diversity with unique evolutionary histories and adaptations (Acosta et al. 2024; Demergasso et al. 2004; Lennartz et al. 2023). This highlights the necessity to preserve these environments, which are currently facing a worldwide decline due to increased droughts, climate change and direct anthropogenic activities like mining or water use (Saccò et al. 2021; Williams 2002; Wurtsbaugh et al. 2017). The increasing salinization of these lakes, or their eventual drying out, could imply the extinction of numerous species, which would result in an irremediable loss for the knowledge of ecological transitions and speciation processes in general.

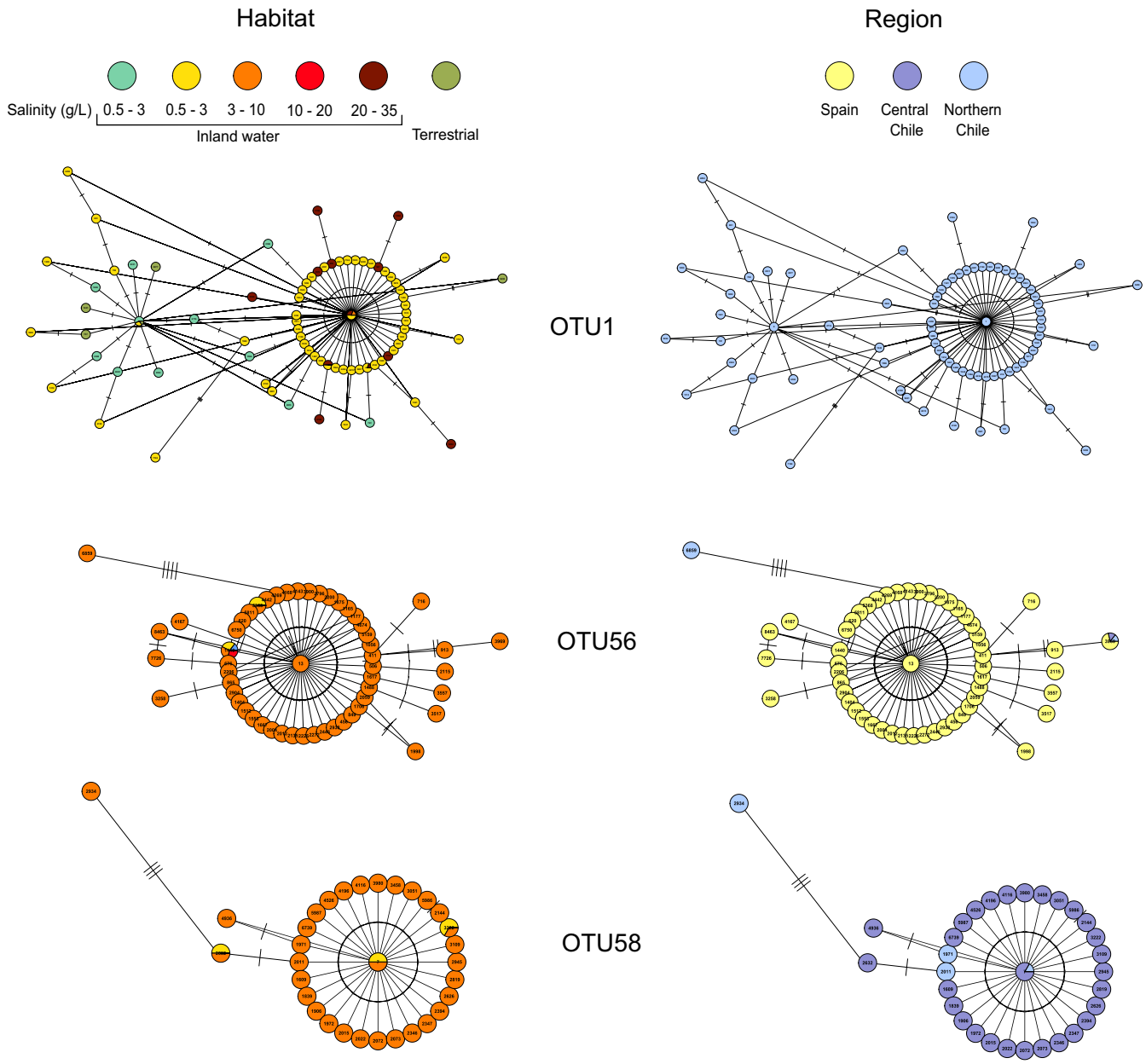

FIGURE 6 | Haplotype networks of OTU1 (above), OTU56 (middle) and OTU58 (below), the OTUs that appeared in more samples and different environments. Each node represents a haplotype, an ASV of that OTU, connected by a line to the closest haplotype, and the marks on the line indicate how many differences are between both sequences. On the left, they are colored by habitat category and on the right by region. The colored portion of the circle indicates the proportion of the category with respect to the total of samples where that ASV was found. Additional networks are provided in Supporting Information S1.

Author Contributions

E.L. conceived and supervised the project. F.U., L.D.F., and E.L. took the samples. F.U., L.D.F., and E.L. carried out microscopy observations. F.U. and C.S.-Z. did the laboratory work. F.U. and R.G.-M. performed the bioinformatics and statistical analysis. All authors discussed the results and contributed to the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The list of samples and associated observations can be found in the Supporting Information S1 section. We also provide an alignment with all the ASVs and barcoded sequence, as well as a maximum likelihood phylogenetic tree including all ASVs. Illumina paired-end sequences

can be accessed in https://www.ncbi.nlm.nih.gov/sra/PRJNA1223952. The bioinformatics pipeline associated to the analyses used in this work is available in Zenodo: 10.5281/zenodo.14007492.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.