ARPHA Preprints

PREPRINT

Author-formatted, not peer-reviewed document posted on 15/04/2025

DOI: https://doi.org/10.3897/arphapreprints.e155725

A comparative analysis of hybridisation capture and PCR-based eDNA metabarcoding for monitoring bryophytes in rivers

Thomas Reinhart, Armando Espinosa Prieto, Thomas Begoc, Hugues Tinguy, Francis Bick, Etienne Chanez, Jean-Nicolas Beisel, Laurent Hardion

ARPHA Preprints Author-formatted, not peer-reviewed document posted on 15/04/2025. DOI: https://doi.org/10.3897/arphapreprints.e155725

A comparative analysis of hybridisation capture and PCR-based eDNA metabarcoding for monitoring bryophytes in rivers

Short title: Comparing eDNA approaches for moss detection in lotic environments

Thomas Reinhart[1*], Armando Espinosa Prieto[1*], Thomas Begoc[1], Hugues Tinguy[2], Francis Bick[2], Etienne Chanez[1], Jean-Nicolas Beisel[1], Laurent Hardion[1]

[1], University of Strasbourg, CNRS, ENGEES, LIVE UMR 7362, 67000 Strasbourg, France

[2], Société Botanique d'Alsace, Sélestat, France

[*], corresponding authors, thomas.reinhart@etu.unistra.fr, armando.espinosa@live-cnrs.unistra.fr

Abstract

Despite their ecological importance, mosses remain under-represented in ecological studies due to their challenging detection in field surveys and morphological identification, exacerbated by the lack of expert botanists. In this study, we optimise an environmental DNA method for the detection of bryophytes from river water samples, with the aim of facilitating their inclusion in biodiversity assessments. We compared three different methods in terms of species detection and community dissimilarity at seven sites along a river. The methods include (i) visual transect surveys conducted by bryologists based on macro- and micro-morphology, (ii) multi-marker PCR metabarcoding of the rbcL and the ITS2 markers with newly designed primers targeting bryophytes, and (iii) hybridisation capture (HC) for the same markers. We found that PCR metabarcoding recovered over 50% (n = 37) of the species observed in the field, whereas hybridization capture detected only 16% (N = 11). PCR metabarcoding identified the most species, 101 species compared to 68 observed in the field and 27 with HC. Both the PCR and HC metabarcoding approaches identified bryophyte species not recorded in field surveys but expected in the catchment. Molecular methods, particularly PCR metabarcoding, recovered elusive and rare species difficult to observed in the field and occurring outside our transect. The two markers used in the molecular approaches contributed uniquely to species detection, making a multi-marker approach necessary to study this group. Environmental DNA and field surveys are complementary methods and together allow the discoverability of inconspicuous species and provide the most exhaustive species inventory.

Keywords: bryophyte-specific primers, freshwater ecosystem, moss, ITS2, *rbc*L, targeted capture

Introduction

Mosses, including Bryophyta, Marchantiophyta, and Antocerotophyta, play a crucial role in ecosystems and biodiversity conservation, especially during the first steps of ecological succession as pioneer species, but also for nutrient cycling (Ayres et al. 2006, Lindo and Gonzalez 2010), soil moisture (Gornall et al. 2007) and water retention (Sharratt 1997). They also serve as a source for microorganisms, while providing habitat for a large diversity of microalgae, fungi, and microfauna (Usher and Booth 1986, Suren 1991, Döbbeler 1997, Roger Anderson 2006, Kauserud et al. 2008). With numerous taxa on red lists (Bick et al. 2014), bryophytes pose significant conservation challenges, further complicated by the scarcity of skilled taxonomists. In fact, they are as threatened as vascular flora, and global change negatively impacts bryophyte colonies (Virtanen et al. 2024). Nonetheless this group is under-represented in ecological studies (Callaghan 2012) and biodiversity conservation policies, particularly because identification to the genus and species levels relies on micro-morphological criteria that requires a high level of expertise (Tinguy 2021). Species

identification is challenging *in situ*, and their small size and low biomass can bias field assessments (Callaghan 2012) and increase reliance on naturalist expertise, risking misidentification (Grace 1995). Molecular identification represents an alternative method that could facilitate the inclusion of these taxa in biodiversity monitoring programs.

To date, bryophytes have only been considered within broader multitaxon surveys, where only a limited number of moss taxa were identified (Cannon et al. 2016, Brunbjerg et al. 2019, Carvalho-Silva et al. 2021, Banerjee et al. 2022, Ariza et al. 2023). The lower biomass of bryophytes compared to vascular plants likely results in reduced eDNA abundance in the environment, potentially hindering their detection in eDNA samples when analysed alongside vascular plants, a challenge further exacerbated by the use of markers and primers primarily designed for flowering plants, as in these studies. To date, there is a no consensus on suitable primers and markers for bryophyte metabarcoding (Liu et al. 2010, Epp et al. 2012, Espinosa Prieto et al. 2023). The few studies targeting bryophyte eDNA used markers within the plastid genome, the *trn*L and *rbc*L (Liu et al. 2010, Von Cräutlein et al. 2011, Ballin et al. 2019, Yodphaka et al. 2018, Nelson et al. 2021). Epp et al. (2012) designed a bryophyte primer pair for ancient DNA studies that amplified c. 50 bp of the *trn*L P6 loop with a taxonomic resolution at the species level of 30% from a database with 4020 species. However, barcode reference databases exhibit a significant gap in sequences for many bryophyte species compared to flowering plants (Liu et al. 2010). The effectiveness of metabarcoding depends on the taxonomic resolution of markers and the universality of primer pairs, that is, their capacity to amplify a broad range of target taxa (Liu et al. 2010, Hassel et al. 2013, Cheng et al. 2016). PCR metabarcoding overamplify taxa with the strongest primer affinity and amplicons with the lower GC content, resulting in the amplification bias that compounds over successive reaction cycles (Moinard et al. 2023).

Hybridisation capture (HC), also known as targeted capture, target enrichment or capture enrichment, is a promising alternative to PCR-based plant eDNA metabarcoding (Foster et al. 2021). Unlike PCR-based metabarcoding, HC circumvents amplification biases, which can lead to false positives and negatives, and uneven species representation (Krehenwinkel et al. 2017, Kelly et al. 2019). The technique relies on biotinylated RNA or DNA molecules (baits), complementary to target DNA regions, to selective capture target sequences while removing non-hybridized DNA (Gnirke et al. 2009, Mamanova et al. 2010, Schuenemann et al. 2011, Carpenter et al. 2013, Marciniak et al. 2015). Hybridisation capture improves species detection accuracy particularly when targeting multiple loci across genomes (Seeber et al. 2019, Jensen et al. 2021, Li et al. 2023). This method is particularly useful for degraded DNA samples, such as ancient DNA (Murchie et al. 2019, Revéret et al. 2023), stool samples (Aylward et al. 2018), and in tropical environments, where it facilitated the detection of low concentrated DNA and low biomass and rare species (Li et al. 2023). Additionally, it reduces PCR failures due to inhibitors co-extracted with eDNA (Jane et al. 2015, Murchie et al. 2019).
This study aims to optimise eDNA methods for bryophyte detection, a taxonomic group underrepresented in ecological research. We compare three methods based on species richness and community dissimilarity. The methods include (i) visual transect surveys conducted by bryologists based on macro- and micro-morphology, (ii) PCR metabarcoding of cpDNA *rbc*L and nrDNA ITS2 markers with newly designed bryophytes-specific primers, and (iii) a hybridisation capture approach for the same markers. The overarching goal is to facilitate the inclusion of bryophytes in ecological assessments.

Methods

Field survey

Our comparative analysis was carried out at seven sites from a previous eDNA study (Espinosa Prieto et al. 2024b) along the Falkensteinerbach River in the Parc naturel regional des Vosges du Nord (Fig. 1). Here, we expand on our previous study in which two moss species were detected as bycatch using eDNA metabarcoding with vascular plant primers. We used the same eDNA extractions (stored at -80°C) from our previous study for the PCR and HC eDNA approaches. Moss inventories were conducted on February 16 and March 8 2024 along a transect parallel to the river's course, with a 5-meter-wide survey zone covering both riverbanks and the riverbed. The survey proceeded from downstream to upstream to minimize sediment disturbance. Bryophyte specimens were collected for species identification using microscopy and barcoding analysis. These samples are preserved in the Herbarium of the University of Strasbourg and in the private collections of Francis Bick and Hugues Tinguy. Species rarity for the study region was obtained from the classification by Tinguy et al. (2021) and used to identify differences in recoverability of rare taxa between methods.

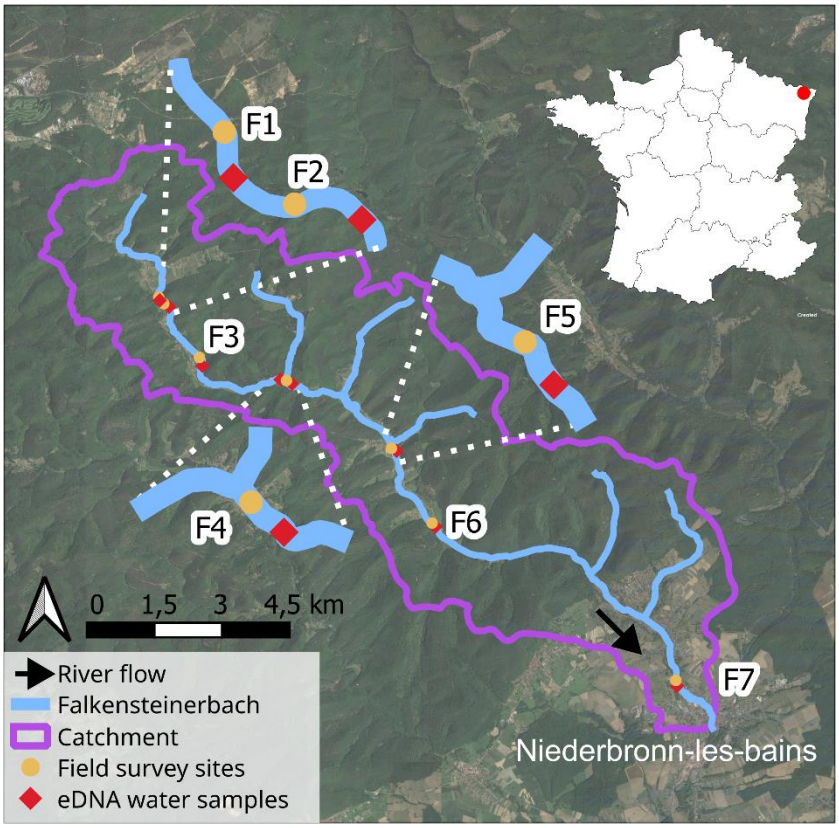

Figure 1. Map of the study area within the Parc naturel regional des Vosges du Nord, showing the seven sites and the seven eDNA sampling locations along the Falkensteinerbach, 1:150000 scale. The map was created in QGIS V3.32.2 with data from the *DataGrandEst Qgis* plug-in.

Designing and adjusting primer pairs for bryophytes

We first reviewed the literature on plant barcoding and metabarcoding to identify suitable primers to amplify the cpDNA *rbc*L gene and the nrDNA internal transcribed spacer 2 (ITS2) in Bryophyta, Marchantiophyta and Anthocerophyta taxa. We querried the Web of Science and PubMed databases in February 2024 (see Table S1, Appendix A for a detailed list of primers). We conducted *in silico* PCRs using ecoPCR from the *OBITools* package (Boyer et al. 2016) to evaluate 17 primer pairs for *rbc*L and 21 for ITS2, allowing up to three mismatches between

primers and binding sites (Fig. 2; Table S2, Appendix A). Amplicon length was set between 100 bp and a marker-specific maximum: 600 bp for ITS2 and 700 bp for *rbc*L. In the absence of a satisfactory primer pair for ITS2 in the literature, we designed a primer pair that preferentially amplifies bryophytes: BraF 5'-CGCAAGTTGCGCCCGAGGCT-3' (forward) and BraR 5'-GTGATATGCTTAAACTCAGCGGG-3' (reverse). We manually explored the alignment and consensus sequences of all available bryophyte ITS2 sequences from the NCBI database using Geneious Prime (Dotmatics, Boston, USA). By comparing the consensus sequence with that of vascular plants, we identified a section within the flanking regions common to all mosses that differed by at least two nucleotides from vascular plants (Fig. 3). Conservation of the priming sites was assessed using the OBITools commands 'ecopcr.forward.shanon' and 'ecopcr.reverse.shanon', and the number of mismatches against mosses and vascular plants was visualised with the command 'mismatchplot'. We applied standard primer design principles, ensuring a melting temperature difference within 5°C between primers, primer length between 18 and 25 nucleotides, and we verified that the melting temperature of primer dimers and hairpin structures exceeded the annealing temperature. Furthermore, we prioritised GC clamps on the 3' end while minimising 3' end mismatches against bryophytes and promoting such mismatches against vascular plants (Fig. S1, Appendix B). The designed primer pair was then subjected to the same *in silico* analyses as the other primers (Fig. S2, Appendix B).

Initial *in silico* analysis of *rbc*L primers from the literature also yielded unsatisfactory results. Unlike for the ITS2, we did not find a better priming site that allowed for the design of metabarcoding primers specific to Bryophytes following the standards above. Instead, we customized primers from the literature (noted *) and rerun the *in silico* analysis. Ten primers were identified, generating amplicons ranging from 107 to 695 bp. Over 95% of species in the reference database were amplified by most primer pairs (barcode coverage, Bc) except for those pairs with the forward 640F or the reverse 804hR (Fig. 4). The three primer pairs containing the forward rbcL_aF primer, which generated amplicons of approximately 500 bp, exhibited the highest species-level barcode specificity (Bs) at around 75% (Fig. 4; Table S3, Appendix A). In contrast, primer pairs producing c. 200 bp amplicons, despite achieving 95% barcode coverage (Bc), demonstrated lower Bs values of approximately 30%, with the highest specificity attributed to primer pairs with the forward rbcL265 (Fig. 4).

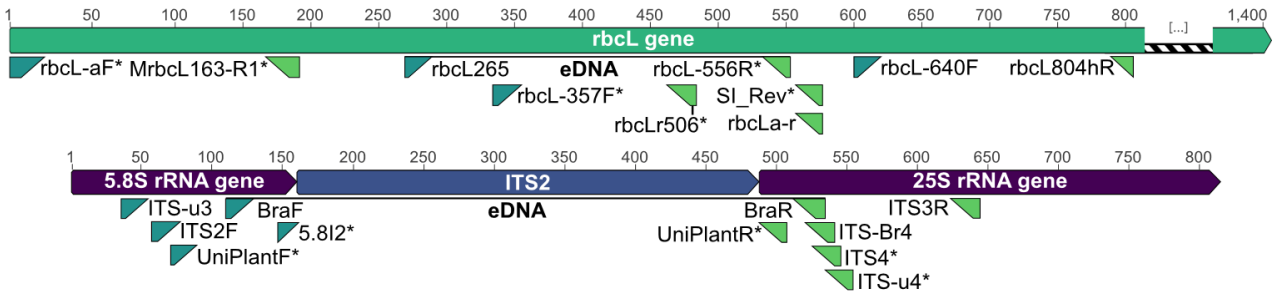

Figure 2. Map of the best primers tested *in silico*. Primers linked with a bar were used for the amplification of eDNA samples.

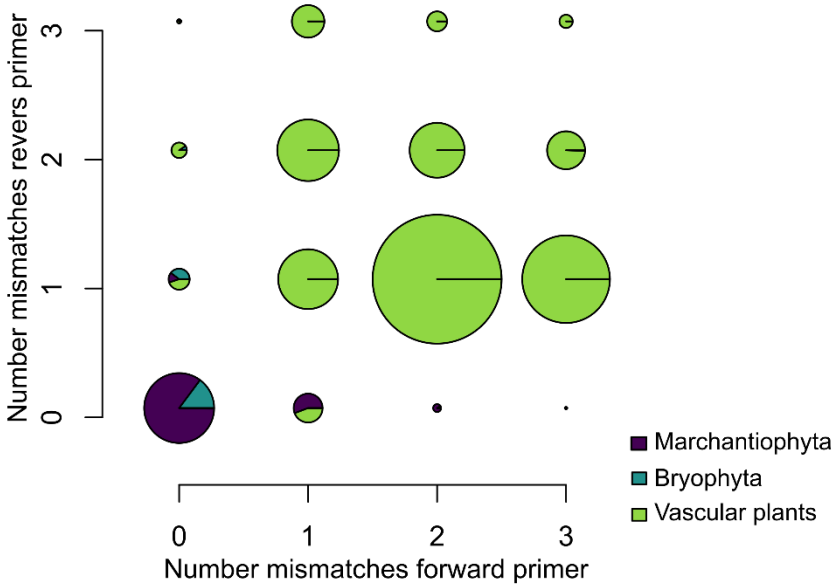

Figure 3. Pie chart depicting the number of mismatches (ranging from zero to three) on the forward primer BRaF (x-axis) and the reverse primer BRaR (y-axis) for the ITS2 marker in mosses and vascular plants. The size of each pie represents the number of sequences successfully amplified *in silico*. Greater mismatch numbers on either primer indicate reduced PCR efficiency.

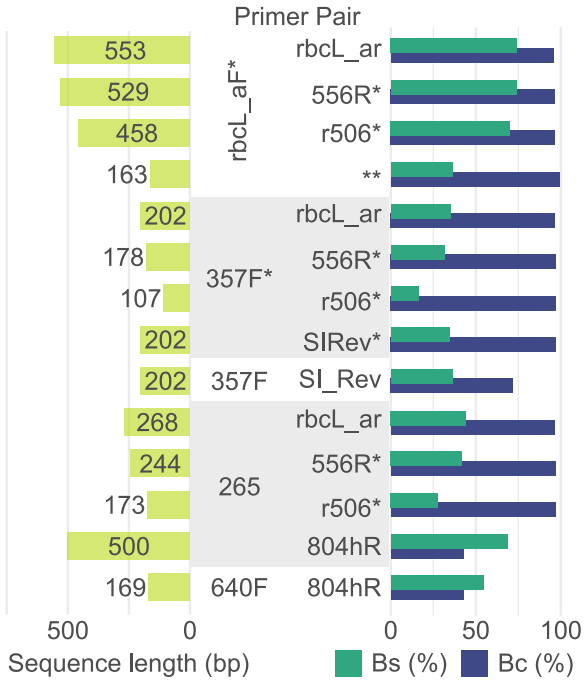

Figure 4. Barcode characteristics of each *rbc*L primer pair tested *in silico*. The mean amplicon length (bp) is shown for each primer pair, with barcode coverage (Bc) and barcode specificity (Bs) are presented as percentages. (*) denotes primers modified in this study from their original sources; (**) indicates the modified reverse primer MrbcL163-R1.

Environmental DNA methods

PCR metabarcoding

The PCR mixtures were prepared under a UV-sterilised hood, cleaned with DNA/RNA-ExitusPlus™ IF (PanReac AppliChem, Germany) and with a Bunsen burner to prevent contamination of DNA samples from the laboratory environment. We used our new primer pair BraF + BraR for ITS2, and for the *rbc*L the primer pair rbcL265* + rbcL556* with our suggested modifications shown in bold 5'-AT**Y**GCTTA**Y**GTTGCTTA**Y**CC-3' (forward) + 5'-CA**Y**T**C**RTA**W**AC**W**GC**T**CTACC-3' (reverse) (modified from Tsubota et al. 1997, Aziz et al. 2017). PCRs were prepared in triplicate using fusion primers with inline dual indexes to tag each sample. PCR reactions were prepared in a final volume of 25 µL following the manufacturer's instructions for the GoTaq® G2 Hot Start Master Mix (Promega, Madison, USA), comprising 12 µL of GoTaq Mmix, 2.5 µL of forward primer, 2.5 µL of reverse primer, 5 µL of DNA, and 3 µL of nuclease-free water. Amplification was carried out for 35 cycles with an initial denaturation at 95°C for 3 min, followed by 98°C for 20 s, Ta=55°C for *rbc*L and Ta=65°C for ITS2 for 15 s, 72°C for 30 s and 72°C for the final elongation time of 1 min. The PCR products were controlled on a 2% agarose gel under UV light and DNA concentrations were measured using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA). Sequencing was performed by Eurofins Genomics Europe (Konstanz, Germany) with Illumina MiSeq 2 x 300 bp chemistry, with a theoretical sequencing depth of 120000 reads per sample. Bioinformatic analysis was performed as in Espinosa Prieto et al. (2024b) adapting the pipeline available in (https://zenodo.org/records/12571296).

Hybridisation capture metabarcoding

The bait set was designed in collaboration with Arbor Biosciences to target the four most common metabarcoding plant markers (*rbc*L, *trn*L, ITS1 and ITS2) based on the curated reference database of Embryophyta plants from the region created for the taxonomic assignment step in our previous study (Espinosa Prieto et al. 2024b) (Appendix C and Appendix D for Krona plot visualisation of the databases). The database comprises 3342 ITS2 sequences of bryophytes, representing 15.5% of the total ITS2 database, and 1043 *rbc*L sequences, accounting for 9% of the *rbc*L database. The bait set consists of c. 19000 baits, each 80mers long, and with three-times tilling, meaning that any given nucleotide is covered by at least three different baits (Murchie et al. 2019). Enrichments were conducted by Daicel Arbor Bioscience (Arbor, USA) following their myBaits v4.1 protocol for each eDNA sample independently, including a blank control consisting of DNA-free Milli-Q water. Each library was sequenced on NovaSeq 2 x 150 bp, generating c. 20 M reads per sample. The reads were trimmed and merged with leeHom (Renaud et al., 2014) using the ancient DNA argument (–ancientdna) as it recovered more sequences than without it and performed a better correction of short reads that contained sequencing adapters. Paired reads were dereplicated using the obiuniq command from OBITools. The site name was added to the sequence headers and the fasta files were then concatenated and dereplicated again on the whole dataset. This file was used as the input for BLASTn against the curated database to return the top 50 alignments per read (unique accession hits) with a minimum of 98% identity (flags: -num_alignments 50 -perc_identity 98). The BLASTn output was then passed to MEGAN (Community Edition, v.6.19.7) (Huson et al., 2007, 2016) where the results were filtered through a lowest common ancestor (LCA) algorithm using unique parameters for each marker: Min score = 100, top percent consideration of hits based on bit score = 3%, LCA weighted algorithm at 80%. The resulting taxonomic assignment was exported and combined with the sequence file, and a custom R script was used to obtain read abundances of the different taxa per site. This table was transformed into presence-absence data considering that a species was present if it had at least four reads for one marker. Multimarker data was created by adding together the tables of *rbc*L and ITS2 markers.

Statistics

The following analysis were performed using R version 4.1.1 (2021.08.10). Venn diagrams were generated using the R package *ggVennDiagram* (version 1.5.2) to compare the proportion of species recovered across methods and to identify differences between markers in the two eDNA approaches. A Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity of community composition (beta diversity) at the same sites was conducted to identify patterns in species detection and assess consistency across methods, using the function *vegdist* from the *vegan* R package (version 2.6-8). We examined whether variations in community composition across methods could be attributed to differences in their sensitivity to detecting species with distinct functional traits, life forms, and ecological niches. Specifically, we assessed the correlation between species detectability by eDNA methods and dispersion traits – defined here as the propensity of taxa to propagate via vegetative propagules or sporophytes as a proxy for higher eDNA abundance – as well as their preference for moist environments. Trait data were obtained from the BET database (Van Zuijlen et al. 2023). Additionally, we conducted an indicator species analysis using the *indcispecies* package in R to evaluate the likelihood of species being preferentially detected by specific methods.

Results

Across all methods, a total of 145 species were detected (see Table S4, Appendix A for a detailed list of taxa); however, only nine species were recovered by all methods (Fig. 5). The field survey identified 68 species, 29 of which were exclusively detected through this approach, including eight species that lacked a reference sequence in the database. PCR metabarcoding recovered over 50% (n = 37) of the species observed in the field, whereas hybridization capture (HC) detected only 16% (n = 11). The latter recovered 27 bryophyte species, with an average of five species per site. Of the nine species only detected by HC (Fig. 5), two are considered very rare in the region, *Dicranella cerviculata* (Hedw.) Schimp. and *Riccia huebeneriana* Lindenb., three are quite rare, *Aneura pinguis* (L.) Dumort., *Hookeria lucens* (Hedw.) Sm. and *Trichocolea tomentella* (Ehrh.) Dumort., three fairly common, *Frullania tamarisci* (L.) Dumort., *Heterocladium heteropterum* (Brid.) Schimp. and *Physcomitrium pyriforme* (Hedw.) Bruch & Schimp., and one is common, *Didymodon luridus* Hornsch. Species recovery differed between the two markers, ITS2 identified three times more species than *rbc*L (Fig. 5) and only two species were detected by both markers (*Fontinalis antipyretica* Hedw. and *Ptychostomum capillare* (Hedw.) Holyoak & N.Pedersen). ITS2 exhibited higher taxonomic resolution than *rbc*L, with 21 out of 31 unique sequences successfully assigned to a species, compared to only 6 out of 12 for *rbc*L, despite both markers having the same amplicon size. For example, 520 reads for the *rbc*L where assigned to Bryaceae at site F6, likely representing multiple taxa from this family observed in the field.

PCR metabarcoding identified 101 species, including 57 species not recovered by the other methods (Fig. 5). Rare species were also detected, for example, *Anomodon rugelii* (Müll.Hal.) Keissl. (Bick et al. 2014, Tinguy 2021) was identified using the *rbc*L marker at sites F4 and F7 (Table S4, Appendix A). Both markers recovered a similar number of species, with rbcL detecting 67 and ITS2 detecting 59 (Fig. 5). However, approximately 40% of the species were uniquely detected by either marker, while only 20% were shared between them. Principal Coordinate Analysis (PCoA) was performed to explore dissimilarity patterns between the three methods. The first two principal coordinates (PC1 and PC2) explained 32.3% and 15.9% of the total variation (Fig. 6). A distinct clustering of samples based to the survey method was observed, indicating methodological biases. However, our analysis refuted the hypothesis that

these biases result from differences in the methods' sensitivity to detecting species with specific traits and ecological preferences, as demonstrated by the rank-sum test on dispersal traits and preference for moist environments. Nevertheless, the indicator species analysis identified a set of species that were more likely to be detected by one method over the other (see Table S5, Appendix A for detailed list of species).

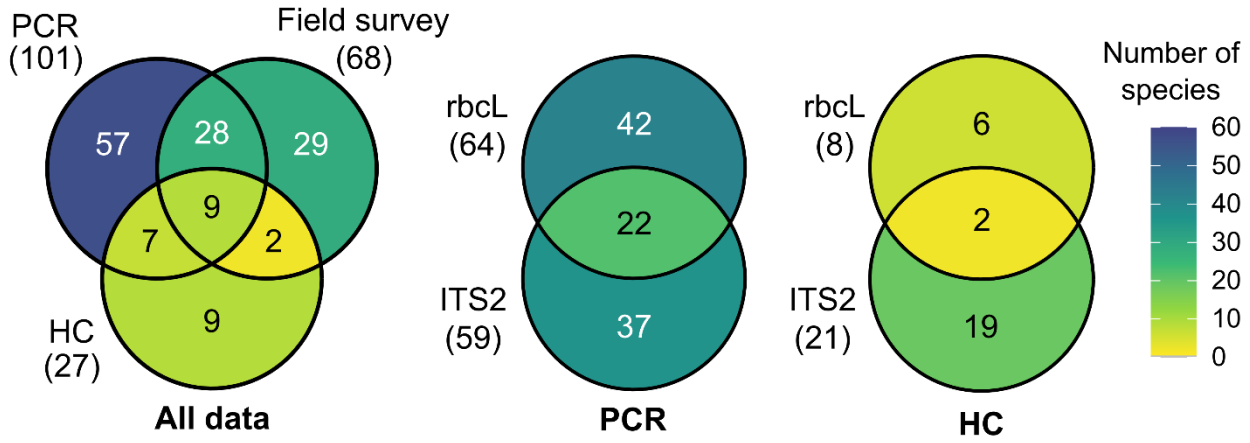

Figure 5. Venn diagrams of the proportion of species for the whole dataset (7 sites) detected through field surveys, PCR metabarcoding and hybridisation capture (HC); and by each marker for the two eDNA approaches. Total species richness (gamma diversity) is shown in parenthesis for each method and marker.

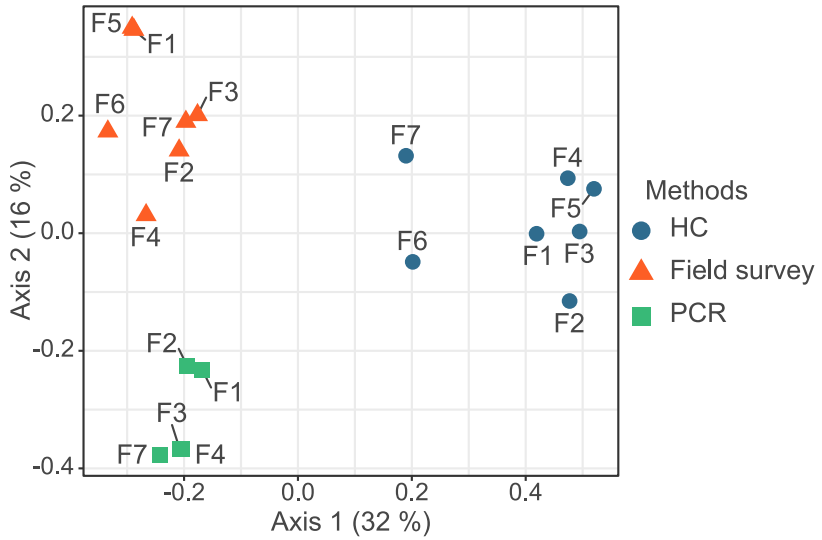

Figure 6. Principal Coordinate Analysis (PCoA) based on Sørensen's similarity index, illustrating differences in community composition. The PCoA compares communities detected by hybridization capture (HC), PCR metabarcoding, and field surveys across all sites.

Discussion

In this study, we compared three different approaches to study bryophyte communities in riverine and riparian environments. Our study is the first to evaluate hybridisation capture and PCR metabarcoding using bryophyte-specific primers, and to compare these to traditional field surveys. PCR metabarcoding has been shown to accurately capture plant species richness and community dissimilarity along ecological gradients (Shackleton et al. 2019, Reji Chacko et al. 2023). However, eDNA metabarcoding in rivers produces substantially different species lists

than field surveys, and studies often highlight the advantage of combining both approaches (Espinosa Prieto et al. 2024b). Our bryophyte specific PCR metabarcoding approach detected over half of the species observed in the field survey, a significant improvement compared to Ariza et al. (2023) who found only a 10% overlap between the methods. However, each method produced different community compositions despite describing the same sites. This discrepancy is common in lotic environment where eDNA overestimates species richness at a point location due to the collection of eDNA from upstream sources (Deiner et al. 2016) while field surveys provide a local estimate of species richness. The recovery of eDNA from river water samples is influenced by its dispersion along the river, spanning from a few meters to several kilometres (Jane et al. 2015, Doi et al. 2021), its retention time in the water column (Harrison et al., 2019), and its resuspension (Shogren et al. 2017). Short fragments can also persist longer in the environment, thus improving spatial and temporal inferences that can be drawn from environmental samples (Bista et al. 2017, Jo et al. 2017). For instance, *Leptobryum pyriforme* (Hedw.) Wilson, 1855 was detected at site F7 through PCR metabarcoding although it was not observed at the site. While this species is unlikely to occur naturally in the region, it is found in synanthropic environments, such as garden pots, arable fields, reservoir margins and damp woods, all of which can be found upstream of this site. Similarly, *Fissidens fontanus* (Bach.Pyl.) Steud., 1824, commonly found in fountains, may have been detected due to runoff water from nearby villages.

In contrast, the rarity of a taxon does not imply its detection by eDNA, for example, *Kindbergia praelonga* (Hedw.) Ochyra, 1982 was observed at all sites, and *Plagiomnium undulatum* (Hedw.) T.J.Kop., 1968 is a widespread species characteristic of riparian habitats. Both species were never detected by eDNA approaches despite having reference barcodes for at least one marker, although the latter species only had one reference sequence for the *rbc*L. Other factors explaining their absence in eDNA records may be from barcode similarity with closely related species, low DNA release rates, and PCR biases. On the contrary, eDNA analysis can detect elusive taxa, such as *Buxbaumia viridis* (Moug. ex Lam. & DC.) Brid. ex Moug. & Nestl. (Annex II of the European Habitats Directive, and strictly protected in France) identified in a nearby watershed with universal plant primers (Espinosa Prieto et al. 2024b) and detected in the present study through bryophyte-specific PCR metabarcoding. This species exhibited read abundances comparable to those of abundant taxa but was not observed in the field, as it persists in the form of protonema which are microscopic undifferentiated chains of cells invisible to the naked eye.

Additionally, we investigated whether biological and ecological traits could explain the observed differences in terms of species detection between the methods. We hypothesised that hygrophilous species, occupying ecological niches closest to the river, would be more likely detected in eDNA water samples than species growing at the edge of our surveys, five meters away from the river. We also hypothesised that species with a propensity to disperse through sporophytes and propagules contributed more to the eDNA signal. We rejected both hypotheses, suggesting that eDNA reflects an integrative signal from both the riverbank and the riverbed. The observed differences in species detection appear to be driven more by the technical limitations of each method than by species-specific characteristics. In particular, barcode specificity and the composition of the reference database influence both eDNA methods, whereas plant size, biomass, and the level of taxonomic expertise affect traditional survey methods.

Marker specificity was a key determinant of species detection in hybridisation capture where the variation observed between markers could be attributed to the limited taxonomic resolution

of short and randomly recovered *rbc*L sequences (150–220 bp). Environmental DNA sequences from the first 200 nucleotides of the *rbc*L gene exhibit lower taxonomic resolution compared to sequences spanning positions 265–500, as demonstrated by the *in silico* barcode specificity analysis using the current bryophyte reference database. Although short DNA fragments persist longer in the environment and are more effectively captured by hybridisation (Li et al. 2023), we observed some non-overlapping read pairs belonging to bryophyte taxa and hinting towards the capture of eDNA fragments above 300 bp. To improve species detection by HC for the *rbc*L, one could use longer sequencing cycles (200 to 250PE). From our results, it is also evident that a multi-marker approach increases species detection in PCR metabarcoding, as is already recommended for vascular plants (Moorhouse-Gann et al. 2018, Da Silva et al. 2019, Espinosa Prieto et al. 2024a). Future studies could also broaden the selection of markers beyond ITS2 and *rbc*L, which have demonstrated specificity for bryophytes in this study and others (Liu et al. 2010, Lang et al. 2014), to include additional markers such as the trnL P6 loop or ITS1. However, efforts should focus on filling the gaps in reference databases which otherwise limit species identification in eDNA approaches.

Conclusions

We demonstrate that eDNA is an effective method for detecting bryophyte species from river water samples. Our comparison of PCR-based and HC metabarcoding highlights the need to explore the methodological biases of eDNA approaches before interpreting ecological patterns. The bryophyte primers designed in this study significantly improved species detection, enhancing the reliability of future studies. In contrast, the capture-based method requires further optimization before considering it for eDNA applications. Nonetheless, molecular and visual surveys remain complementary to each other for recovering the riparian and riverine bryophyte community.

Acknowledgments

We are grateful to the Conservatoire Botanique d'Alsace Lorraine, the Société Botanique d'Alsace, and the Parc Naturel Régional des Vosges du Nord (PNRVN) for providing naturalist data. We also thank the PNRVN for granting us access to the study sites. This study stems from previous work and data collected as part of the Interreg EcoServ program (https://project-ecoserv.eu). Financial support for this project was provided by the École Nationale du Génie de l'Eau et de l'Environnement de Strasbourg and the University of Strasbourg through the IdEx—Initiative d'excellence, project CaptADN. The authors would like to acknowledge the High Performance Computing Center of the University of Strasbourg for supporting this work by providing scientific support and access to computing resources. Part of the computing resources were funded by the Equipex Equip@Meso project (Programme Investissements d'Avenir) and the CPER Alsacalcul/Big Data.

References

Ariza M, Fouks B, Mauvisseau Q, Halvorsen R, Alsos IG, De Boer HJ (2023) Plant biodiversity assessment through soil eDNA reflects temporal and local diversity. Methods in Ecology and Evolution 14: 415–430. https://doi.org/10.1111/2041-210X.13865

Aylward ML, Sullivan AP, Perry GH, Johnson SE, Louis EE (2018) An environmental DNA sampling method for aye-ayes from their feeding traces. Ecology and Evolution 8: 9229–9240. https://doi.org/10.1002/ece3.4341

Ayres E, Van Der Wal R, Sommerkorn M, Bardgett RD (2006) Direct uptake of soil nitrogen by mosses. Biology Letters 2: 286–288. https://doi.org/10.1098/rsbl.2006.0455

Aziz SA, Clements GR, Peng LY, Campos-Arceiz A, McConkey KR, Forget P-M, Gan HM (2017) Elucidating the diet of the island flying fox (*Pteropus hypomelanus*) in Peninsular Malaysia through Illumina Next-Generation Sequencing. PeerJ 5: e3176. https://doi.org/10.7717/peerj.3176

Ballin NZ, Onaindia JO, Jawad H, Fernandez-Carazo R, Maquet A (2019) High-resolution melting of multiple barcode amplicons for plant species authentication. Food Control 105: 141–150. https://doi.org/10.1016/j.foodcont.2019.05.022

Banerjee P, Stewart KA, Dey G, Antognazza CM, Sharma RK, Maity JP, Saha S, Doi H, De Vere N, Chan MWY, Lin P-Y, Chao H-C, Chen C-Y (2022) Environmental DNA analysis as an emerging non-destructive method for plant biodiversity monitoring: a review. Ohgushi T (Ed.). AoB PLANTS 14: plac031. https://doi.org/10.1093/aobpla/plac031

Bick F, Stoehr B, Heuacker V (2014) La Liste rouge des Bryophytes menacées en Alsace. SBA, ODONAT: 55. Available from: https://www.odonat-grandest.fr/wp-content/uploads/2017/12/LR_Bryophytes_Alsace_2014.pdf

Bista I, Carvalho GR, Walsh K, Seymour M, Hajibabaei M, Lallias D, Christmas M, Creer S (2017) Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. Nature Communications 8: 14087. https://doi.org/10.1038/ncomms14087

Boyer F, Mercier C, Bonin A, Le Bras Y, Taberlet P, Coissac E (2016) OBITOOLS : a UNIX - inspired software package for DNA metabarcoding. Molecular Ecology Resources 16: 176–182. https://doi.org/10.1111/1755-0998.12428

Brunbjerg AK, Bruun HH, Brøndum L, Classen AT, Dalby L, Fog K, Frøslev TG, Goldberg I, Hansen AJ, Hansen MDD, Høye TT, Illum AA, Læssøe T, Newman GS, Skipper L, Søchting U, Ejrnæs R (2019) A systematic survey of regional multi-taxon biodiversity: evaluating strategies and coverage. BMC Ecology 19: 43. https://doi.org/10.1186/s12898-019-0260-x

Callaghan D (2012) The inventory of bryophytes at sites: completeness and survey effort. Journal of Bryology 34: 37–44. https://doi.org/10.1179/1743282011Y.0000000046

Cannon MV, Hester J, Shalkhauser A, Chan ER, Logue K, Small ST, Serre D (2016) In silico assessment of primers for eDNA studies using PrimerTree and application to characterize the biodiversity surrounding the Cuyahoga River. Scientific Reports 6. https://doi.org/10.1038/srep22908

Carpenter ML, Buenrostro JD, Valdiosera C, Schroeder H, Allentoft ME, Sikora M, Rasmussen M, Gravel S, Guillén S, Nekhrizov G, Leshtakov K, Dimitrova D, Theodossiev N, Pettener D, Luiselli D, Sandoval K, Moreno-Estrada A, Li Y, Wang J, Gilbert MTP, Willerslev E, Greenleaf WJ, Bustamante CD (2013) Pulling out the 1%: Whole-Genome Capture for the Targeted Enrichment of Ancient DNA Sequencing Libraries. American Journal of Human Genetics 93: 852. https://doi.org/10.1016/j.ajhg.2013.10.002

Carvalho-Silva M, Rosa LH, Pinto OHB, Da Silva TH, Henriques DK, Convey P, Câmara PEAS (2021) Exploring the plant environmental DNA diversity in soil from two sites on Deception Island (Antarctica, South Shetland Islands) using metabarcoding. Antarctic Science 33: 469–478. https://doi.org/10.1017/S0954102021000274

Cheng T, Xu C, Lei L, Li C, Zhang Y, Zhou S (2016) Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. Molecular Ecology Resources 16: 138–149. https://doi.org/10.1111/1755-0998.12438

Da Silva LP, Mata VA, Lopes PB, Pereira P, Jarman SN, Lopes RJ, Beja P (2019) Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic

generalists. Molecular Ecology Resources 19: 1420–1432. https://doi.org/10.1111/1755-0998.13060

Deiner K, Fronhofer EA, Mächler E, Walser J-C, Altermatt F (2016) Environmental DNA reveals that rivers are conveyer belts of biodiversity information. Nature Communications 7: 12544. https://doi.org/10.1038/ncomms12544

Döbbeler P (1997) Biodiversity of bryophilous ascomycetes. Biodiversity and Conservation 6: 721–738. https://doi.org/10.1023/A:1018370304090

Doi H, Akamatsu Y, Goto M, Inui R, Komuro T, Nagano M, Minamoto T (2021) Broad-scale detection of environmental DNA for an invasive macrophyte and the relationship between DNA concentration and coverage in rivers. Biological Invasions 23: 507–520. https://doi.org/10.1007/s10530-020-02380-9

Epp LS, Boessenkool S, Bellemain EP, Haile J, Esposito A, Riaz T, Erséus C, Gusarov VI, Edwards ME, Johnsen A, Stenøien HK, Hassel K, Kauserud H, Yoccoz NG, Bråthen KA, Willerslev E, Taberlet P, Coissac E, Brochmann C (2012) New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. Molecular Ecology 21: 1821–1833. https://doi.org/10.1111/j.1365-294X.2012.05537.x

Espinosa Prieto A, Beisel J-N, Verschuren P, Hardion L (2023) Toward freshwater plant diversity surveys with eDNA barcoding and metabarcoding. Environmental DNA 5: 648–670. https://doi.org/10.1002/edn3.407

Espinosa Prieto A, Hardion L, Debortoli N, Beisel J (2024a) Finding the perfect pairs: A matchmaking of plant markers and primers for multi-marker eDNA metabarcoding. Molecular Ecology Resources: e13937. https://doi.org/10.1111/1755-0998.13937

Espinosa Prieto A, Hardion L, Debortoli N, Bournonville T, Mathot T, Marescaux J, Chanez E, Staentzel C, Beisel J-N (2024b) A comparative analysis of eDNA metabarcoding and field surveys: Exploring freshwater plant communities in rivers. Science of The Total Environment 954: 176200. https://doi.org/10.1016/j.scitotenv.2024.176200

Foster NR, Van Dijk K, Biffin E, Young JM, Thomson VA, Gillanders BM, Jones AR, Waycott M (2021) A Multi-Gene Region Targeted Capture Approach to Detect Plant DNA in Environmental Samples: A Case Study From Coastal Environments. Frontiers in Ecology and Evolution 9: 735744. https://doi.org/10.3389/fevo.2021.735744

Gnirke A, Melnikov A, Maguire J, Rogov P, LeProust EM, Brockman W, Fennell T, Giannoukos G, Fisher S, Russ C, Gabriel S, Jaffe DB, Lander ES, Nusbaum C (2009) Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. Nature Biotechnology 27: 182–189. https://doi.org/10.1038/nbt.1523

Gornall JL, Jónsdóttir IS, Woodin SJ, Van Der Wal R (2007) Arctic mosses govern below-ground environment and ecosystem processes. Oecologia 153: 931–941. https://doi.org/10.1007/s00442-007-0785-0

Grace M (1995) A key to the growthforms of mosses and liverworts and guide to their educational value. Journal of Biological Education 29: 272–278. https://doi.org/10.1080/00219266.1995.9655460

Harrison JB, Sunday JM, Rogers SM (2019) Predicting the fate of eDNA in the environment and implications for studying biodiversity. Proceedings of the Royal Society B: Biological Sciences 286: 20191409. https://doi.org/10.1098/rspb.2019.1409

Hassel K, Segreto R, Ekrem T (2013) Restricted variation in plant barcoding markers limits identification in closely related bryophyte species. Molecular Ecology Resources 13: 1047–1057. https://doi.org/10.1111/1755-0998.12074

Huson DH, Auch AF, Qi J, Schuster SC (2007) MEGAN analysis of metagenomic data. Genome Research 17: 377–386. https://doi.org/10.1101/gr.5969107

Huson DH, Beier S, Flade I, Górska A, El-Hadidi M, Mitra S, Ruscheweyh H-J, Tappu R (2016) MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. Poisot T (Ed.). PLOS Computational Biology 12: e1004957. https://doi.org/10.1371/journal.pcbi.1004957

Jane SF, Wilcox TM, McKelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR (2015) Distance, flow and PCR inhibition: e DNA dynamics in two headwater streams. Molecular Ecology Resources 15: 216–227. https://doi.org/10.1111/1755-0998.12285

Jensen MR, Sigsgaard EE, Liu S, Manica A, Bach SS, Hansen MM, Møller PR, Thomsen PF (2021) Genome-scale target capture of mitochondrial and nuclear environmental DNA from water samples. Molecular Ecology Resources 21: 690–702. https://doi.org/10.1111/1755-0998.13293

Jo T, Murakami H, Masuda R, Sakata MK, Yamamoto S, Minamoto T (2017) Rapid degradation of longer DNA fragments enables the improved estimation of distribution and biomass using environmental DNA. Molecular Ecology Resources 17. https://doi.org/10.1111/1755-0998.12685

Kauserud H, Mathiesen C, Ohlson M (2008) High diversity of fungi associated with living parts of boreal forest bryophytes. Botany 86: 1326–1333. https://doi.org/10.1139/B08-102

Kelly RP, Shelton AO, Gallego R (2019) Understanding PCR Processes to Draw Meaningful Conclusions from Environmental DNA Studies. Scientific Reports 9: 12133. https://doi.org/10.1038/s41598-019-48546-x

Krehenwinkel H, Wolf M, Lim JY, Rominger AJ, Simison WB, Gillespie RG (2017) Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. Scientific Reports 7: 17668. https://doi.org/10.1038/s41598-017-17333-x

Lang AS, Kruijer JD, Stech M (2014) DNA barcoding of Arctic bryophytes: an example from the moss genus Dicranum (Dicranaceae, Bryophyta). Polar Biology 37: 1157–1169. https://doi.org/10.1007/s00300-014-1509-7

Li J, Seeber P, Axtner J, Crouthers R, Groenenberg M, Koehncke A, Courtiol A, Chanratana P, Greenwood AD (2023) Monitoring terrestrial wildlife by combining hybridization capture and metabarcoding data from waterhole environmental DNA. Biological Conservation 284: 110168. https://doi.org/10.1016/j.biocon.2023.110168

Lindo Z, Gonzalez A (2010) The Bryosphere: An Integral and Influential Component of the Earth's Biosphere. Ecosystems 13: 612–627. https://doi.org/10.1007/s10021-010-9336-3

Liu Y, Yan H, Cao T, Ge X (2010) Evaluation of 10 plant barcodes in Bryophyta (Mosses). Journal of Systematics and Evolution 48: 36–46. https://doi.org/10.1111/j.1759-6831.2009.00063.x

Mamanova L, Coffey AJ, Scott CE, Kozarewa I, Turner EH, Kumar A, Howard E, Shendure J, Turner DJ (2010) Target-enrichment strategies for next-generation sequencing. Nature Methods 7: 111–118. https://doi.org/10.1038/nmeth.1419

Marciniak S, Klunk J, Devault A, Enk J, Poinar HN (2015) Ancient human genomics: the methodology behind reconstructing evolutionary pathways. Journal of Human Evolution 79: 21–34. https://doi.org/10.1016/j.jhevol.2014.11.003

Moinard S, Piau D, Laporte F, Rioux D, Taberlet P, Gonindard-Melodelima C, Coissac E (2023) Towards quantitative DNA Metabarcoding: A method to overcome PCR amplification bias. https://doi.org/10.1101/2023.10.03.560640

Moorhouse-Gann RJ, Dunn JC, De Vere N, Goder M, Cole N, Hipperson H, Symondson WOC (2018) New universal ITS2 primers for high-resolution herbivory analyses using DNA metabarcoding in both tropical and temperate zones. Scientific Reports 8: 8542. https://doi.org/10.1038/s41598-018-26648-2

Murchie TJ, Monteath AJ, Mahony ME, Long GS, Cocker S, Sadoway T, Karpinski E, Zazula G, MacPhee RDE, Froese D, Poinar HN (2021) Collapse of the mammoth-steppe in central Yukon as revealed by ancient environmental DNA. Nature Communications 12: 7120. https://doi.org/10.1038/s41467-021-27439-6

Murchie TJ, Kuch M, Duggan AT, Ledger ML, Roche K, Klunk J, Karpinski E, Hackenberger D, Sadoway T, MacPhee R, Froese D, Poinar H (2019) Optimizing extraction and targeted capture of ancient environmental DNA for reconstructing past environments using the PalaeoChip Arctic-1.0 bait-set. Quaternary Research 99: 305–328. https://doi.org/10.1017/qua.2020.59

Nelson JM, Hauser DA, Li F (2021) The diversity and community structure of symbiotic cyanobacteria in hornworts inferred from long-read amplicon sequencing. American Journal of Botany 108: 1731–1744. https://doi.org/10.1002/ajb2.1729

Reji Chacko M, Altermatt F, Fopp F, Guisan A, Keggin T, Lyet A, Rey P-L, Richards E, Valentini A, Waldock C, Pellissier L (2023) Catchment-based sampling of river eDNA integrates terrestrial and aquatic biodiversity of alpine landscapes. Oecologia. https://doi.org/10.1007/s00442-023-05428-4

Renaud G, Stenzel U, Kelso J (2014) leeHom: adaptor trimming and merging for Illumina sequencing reads. Nucleic Acids Research 42: e141–e141. https://doi.org/10.1093/nar/gku699

Revéret A, Rijal DP, Heintzman PD, Brown AG, Stoof-Leichsenring KR, Alsos IG (2023) Environmental DNA of aquatic macrophytes: The potential for reconstructing past and present vegetation and environments. Freshwater Biology 68: 1929–1950. https://doi.org/10.1111/fwb.14158

Roger Anderson O (2006) The Density and Diversity of Gymnamoebae Associated with Terrestrial Moss Communities (Bryophyta: Bryopsida) in a Northeastern U.S. Forest. Journal of Eukaryotic Microbiology 53: 275–279. https://doi.org/10.1111/j.1550-7408.2006.00103.x

Schuenemann VJ, Bos K, DeWitte S, Schmedes S, Jamieson J, Mittnik A, Forrest S, Coombes BK, Wood JW, Earn DJD, White W, Krause J, Poinar HN (2011) Targeted enrichment of ancient pathogens yielding the pPCP1 plasmid of Yersinia pestis from victims of the Black Death. Proceedings of the National Academy of Sciences 108: E746–E752. https://doi.org/10.1073/pnas.1105107108

Seeber PA, McEwen GK, Löber U, Förster DW, East ML, Melzheimer J, Greenwood AD (2019) Terrestrial mammal surveillance using hybridization capture of environmental DNA from African waterholes. Molecular Ecology Resources 19: 1486–1496. https://doi.org/10.1111/1755-0998.13069

Shackleton ME, Rees GN, Watson G, Campbell C, Nielsen D (2019) Environmental DNA reveals landscape mosaic of wetland plant communities. Global Ecology and Conservation 19. https://doi.org/10.1016/j.gecco.2019.e00689

Sharratt BS (1997) Thermal conductivity and water retention of a black spruce forest floor. Soil Science 162. Available from: https://journals.lww.com/soilsci/fulltext/1997/08000/thermal__conductivity_and_water_retention_of_a.6.aspx

Shogren AJ, Tank JL, Andruszkiewicz E, Olds B, Mahon AR, Jerde CL, Bolster D (2017) Controls on eDNA movement in streams: Transport, Retention, and Resuspension. Scientific Reports 7: 5065. https://doi.org/10.1038/s41598-017-05223-1

Suren AM (1991) Bryophytes as invertebrate habitat in two New Zealand alpine streams. Freshwater Biology 26: 399–418. https://doi.org/10.1111/j.1365-2427.1991.tb01407.x

Tinguy H (2021) Catalogue des bryophytes du Bas Rhin (France). Les Nouvelles Archives de la Flore jurassienne et du nord-est de la France 19: 117. Available from: https://cbnfc-ori.org/especes-vegetales/catalogue-des-bryophytes-du-bas-rhin-france.

Tsubota H, Nakao N, Arikawa T, Yamaguchi T, Higuchi M, Deguchi H, Seki T (1997) A preliminary phylogeny of Hypnales (Musci) as inferred from chloroplast rbcL sequence data. Bryological Research 7: 233–248. https://doi.org/10.24474/bryologicalresearch.7.8_233

Usher MB, Booth RG (1986) Arthropod Communities in a Maritime Antarctic Moss-Turf Habitat: Multiple Scales of Pattern in the Mites and Collembola. The Journal of Animal Ecology 55: 155. https://doi.org/10.2307/4699

Van Zuijlen K, Nobis MP, Hedenäs L, Hodgetts N, Calleja Alarcón JA, Albertos B, Bernhardt-Römermann M, Gabriel R, Garilleti R, Lara F, Preston CD, Simmel J, Urmi E, Bisang I, Bergamini A (2023) Bryophytes of Europe Traits (BET) data set: A fundamental tool for ecological studies. Journal of Vegetation Science 34: e13179. https://doi.org/10.1111/jvs.13179

Virtanen R, Harpole WS, Dunker S, Eskelinen A (2024) Multiple global change factors cause declines of a temperate bryophyte. Plant Ecology & Diversity: 1–12. https://doi.org/10.1080/17550874.2024.2330659

Von Cräutlein M, Korpelainen H, Pietiläinen M, Rikkinen J (2011) DNA barcoding: a tool for improved taxon identification and detection of species diversity. Biodiversity and Conservation 20: 373–389. https://doi.org/10.1007/s10531-010-9964-0

Yodphaka S, Boonpragob K, Lumbsch HT, Kraichak E (2018) Evaluation of six regions for their potential as DNA barcodes in epiphyllous liverworts from Thailand. Applications in Plant Sciences 6: e01174. https://doi.org/10.1002/aps3.1174