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RESEARCH ARTICLE

Uncovering the hidden biodiversity of streams at the upper distribution limit of fish

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Abstract

Aim: Biodiversity changes can occur gradually or as distinct jumps as species distributions end and others begin. However, comprehensive assessments of freshwater biodiversity and how assemblages change along a continuum have been hindered by the connectivity and networked nature of streams and their large numbers of rare and cryptic species found there. We hypothesize that the boundary where fish distributions end marks an ecological shift in freshwater assemblages and could unify stream concepts.

Location: Trask River watershed, Oregon, USA, temperate North America.

Taxon: Fishes, amphibians, crayfishes, bivalves, mammals, pathogens.

Methods: We targeted freshwater species across taxa using multigene eDNA metabarcoding of 48 different DNA barcodes to identify assemblages to understand species patterns distributed along a longitudinal gradient in watersheds around the upper fish distribution boundary. Detections from multiple genetic sources improve the detection probability for rare species.

Results: We detected a shift in the assemblage from lower reaches towards headwaters where fish distributions end, leading to fewer species across taxa at reduced eDNA signal. We uncovered a hidden biodiversity in watersheds by detecting a sparsely distributed amphibian pathogen *Batrachochytrium dendrobatidis*, the elusive mammal Mountain Beaver, fish species that went undetected using traditional approaches, and newly discovered cryptic lineages of sculpins. Salmonids were detected further upstream with eDNA than expected.

Main conclusions

Our findings unify stream concepts owing to the marked species shift at the upper extent of fish elevating the importance of this boundary. The detection of salmonids further upstream suggests a distribution extension. The sculpin detections reveal a cryptic species complex with ramifications for fish conservation, potential pockets of endemism and possible identification of new species. We show extensive diversity in watersheds, some of which was previously hidden, transforming our understanding of species presence and distributions affecting our ability to track, protect and manage them across watersheds.

KEYWORDS
cryptic species, eDNA metabarcoding, fish zones, freshwater biodiversity, hidden biodiversity, last fish, river continuum concept, streams, upper extent of fish, watersheds

1 | INTRODUCTION

Ecologists have long recognized the importance of understanding the distribution of organisms for conservation and management decision-making, as this information allows us to describe the biodiversity in an area. However, delimiting distributions of freshwater organisms in streams is challenging, owing to the relatively small area of the globe that comprises freshwater habitats (<1%; Gleick, 1998), the high degree of connectivity of freshwaters (Revenga et al., 2005), the networked nature of streams and rivers (Benda et al., 2004) and the large numbers of rare, endemic and cryptic species found in these habitats (Collen et al., 2014). Understanding the distributions and biogeographic connectivity of freshwater organisms in streams enables us to quantify the spatial variation in freshwater biodiversity, determine which species can adapt to change from stressors responsible for global biodiversity declines (Dudgeon et al., 2006; Reid et al., 2019) and identify ideas that improve our understanding of stream ecology and patterns of freshwater biodiversity. A global agenda for advancing freshwater biodiversity research has been presented, marking innovative methods for biodiversity assessments as a top priority, including methods based on DNA (Maasri et al., 2022).

Ecological concepts on stream biodiversity through the 1970s considered streams to be a series of zones, largely defined by the dominant fishes (fish zones, Shelford, 1911). However, the river continuum concept challenged many of these ideas, predicting shifts in distributions of biota along the gradient of a stream, aligned with measurable changes in channel condition (Vannote et al., 1980). Since 1980, streams have been seen as connected waterways along a patchy and heterogeneous continuum resulting from longitudinal (Vannote et al., 1980), lateral (Gregory et al., 1991; Junk et al., 1989) and vertical processes (Boulton et al., 1998) largely governed by climate, flow, geomorphology and human influences. Accordingly, subsequent studies explored this concept with species distribution data, predicting either continuous shifts in assemblages along a longitudinal stream gradient (Vannote et al., 1980) or distinct jumps at critical points (Montgomery, 1999).

Stream assemblages change from headwaters through their river network and into estuaries, but details about how the change occurs are less understood. Most studies have found an upstream–downstream continuum gradient for fishes, stream-living amphibians and crayfishes (Oberdorff et al., 1993; Římalová et al., 2014; Wilkins & Peterson, 2000), attributed to the continuous movement of material, energy and species longitudinally. Others have found distinct jumps for species (McGarvey & Hughes, 2008; Taylor, 1983; Welsh Jr. & Ollivier, 1998) supporting predictable and distinct transitions in species distributions. Patterns in distributions of fishes, stream-living amphibians, crayfishes, mussels and freshwater assemblages have been attributed to stream size (Oberdorff et al., 1993; Wahbe & Bunnell, 2003), river section (lower, middle and upper; McGarvey & Hughes, 2008), habitat type (pool or riffle) or local habitat conditions, including gradient (Mazzoni et al., 2006). A suite of human-caused factors can also affect species distributions and assemblages, including pollution (Oberdorff et al., 1993), dams (Barnett & Adams, 2021) and riparian habitat management (Olson & Ares, 2022).

We hypothesize that the distribution limit where fish distributions end likely marks an ecological shift in freshwater assemblages and may be the nexus in the stream networks that unifies stream concepts. If species drop out gradually along an upstream–downstream gradient in streams, there would be support for the river continuum concept (Vannote et al., 1980). However, if species end abruptly at a jump, then it would support stream concepts related to zones marking a critical point or ecological shift in streams. We propose that the upper extent of fish species marks a nexus that unifies both stream concepts where the intersection of abiotic factors of stream size, slope and elevation converges to mark the geophysical point in a stream above which no more fishes are found (Penaluna et al., 2022), while other taxa continue to be distributed along a stream continuum. Consequently, questions remain about the spatial heterogeneity of freshwater biodiversity across the transition from below to above the distribution of fish.

Here, we evaluate the spatial variation in freshwater biodiversity of competing expectations of species occurrences and assemblages as either being a continuous gradient or a jump across four neighbouring watersheds, as measured by multigene eDNA metabarcoding. At the downstream-most site in each watershed, we compared the detection of fishes and amphibians using eDNA of stream water to backpack electrofishing. To evaluate shifts in species occurrence and assemblages along a stream continuum, we collected multigene eDNA metabarcoding information from seven additional sites extending upstream within each watershed including for overlooked and less-studied taxa such as crayfishes, bivalves and pathogens. Our goal was to tap into the massive amount of biodiversity information moving through streams using new genomic tools (Deiner et al., 2017; Valentini et al., 2016) to understand species occurrences and assemblages along a stream continuum.

2 | MATERIALS AND METHODS

2.1 | Study area

We evaluated four headwater watersheds of the Trask River: Gus Creek, Pothole Creek, Rock Creek and the upper mainstem Trask, in a temperate forested region of the Oregon Coast Range, USA (Figure 1). These watersheds are managed by Weyerhaeuser

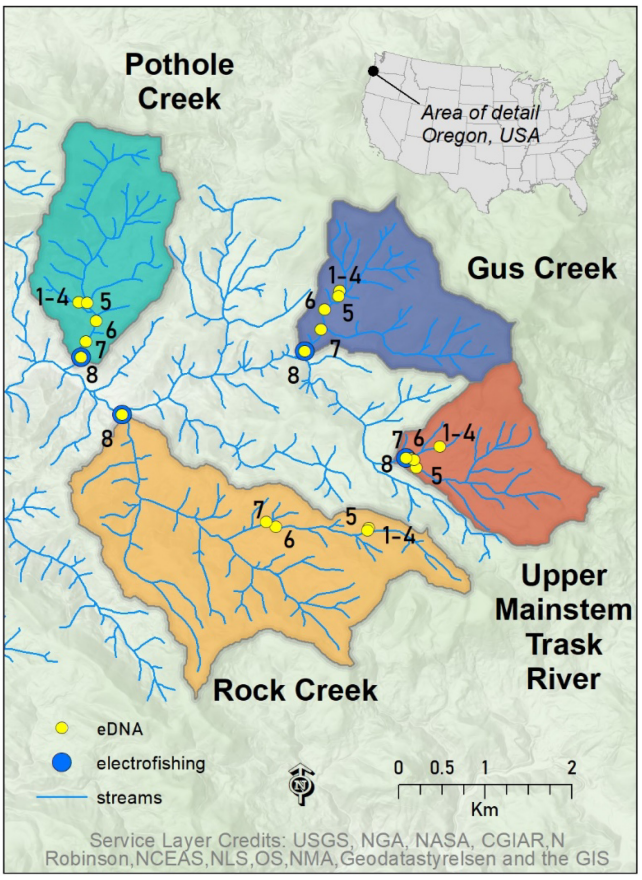

FIGURE 1 Study area map of four neighbouring watersheds in north-western Oregon, including Gus, Pothole and Rock creeks, and upper mainstem Trask River. The site furthest upstream is referred to as '1' and the site furthest downstream is '8'. Electrofishing occurred at site 8. eDNA samples were taken at sites 1–8.

Company, the US Bureau of Land Management and Oregon Department of Forestry. Riparian and upslope vegetation is entirely second-growth forests that grew back following a series of large fires from 1933 to 1951. Portions of upstream reaches in all watersheds except Rock Creek were harvested in 2012. Climate is characterized by mild temperatures, dry summers and wet winters. Precipitation averages 200 cm per year with most precipitation falling as rain between October and May, and occasional snow at higher elevations.

2.2 | Field sampling: Electrofishing sampling and water collection

We conducted backpack electrofishing for fishes and amphibians in an ~300-m reach at the most-downstream site, site 8, in each of the neighbouring watershed in late July of 2016 (Jensen, 2017). To ensure closure for population estimates, we installed block nets at the upper and lower reach-ends while sampling. We marked individuals during an initial mark pass, followed by a recapture pass 1–5 days later. We counted and identified animals to species, except young-of-year (YOY) trout (<50 mm) and Riffle and Reticulate Sculpin (*Cottus gulosus*; *C. perplexus*), which cannot be morphologically distinguished with certainty in the field.

Four eDNA sampling sites were clustered high in each watershed and four lower in each watershed along a stream continuum. The site furthest upstream was labelled as '1' and the site furthest downstream site as '8' (Figure 1). Site 8 is the same site named the 'downstream site' for the Trask watershed study (https://traskstudy.forestry.oregonstate.edu). We sampled during seasonal low flow on 4–23 August 2016, collecting 3-L water samples (2-L thalweg +1-L adjacent slack water) at each site per replicate. Samples were collected in triplicate (e.g. Goldberg et al., 2016). We pumped sample water through 0.45-micron single–use cellulose nitrate filters (Sterlitech, Kent, WA, USA) using a vacuum pump. To prevent cross-site contamination, we entered sites downstream from where samples were taken, and equipment (bottles, tweezers, waders) was decontaminated with a 50% bleach solution followed by a triple rinse of deionized water. Filters were loosely rolled, stored frozen in 5-mL vials on wet ice during collection and transport and then frozen at −20°C within 6 hours of collection until DNA extraction. DNA was extracted from each filter using MoBio's PowerWater© DNA isolation kit (Qiagen, Hilden, Germany) per manufacturer's instructions. Post-extraction samples were cleaned and concentrated using the Zymoclean© Large Fragment DNA Recovery Kit (Zymo Research, Irvine, CA, USA).

2.3 | eDNA amplification and sequence analysis pipeline

Multigene eDNA metabarcoding makes taxonomic identifications using multiple independent primers targeting different genes, allowing for detections from multiple sources and improving detection probability for rare species. We used 48 different DNA barcodes with simultaneous PCR amplification in a single sample to combine 'taxon-general' (e.g. orders, families, genera) and 'taxon-specific' (e.g. genera, species) screening methods (Hauck et al., 2019). We also created three new primer sets to increase and expand the detection of amphibians and sculpins (see Table S1 for complete primer list for this study). Our negative and positive controls also followed the methods from Hauck et al. (2019), in which we created a multi-target standard that included either 2×10^6 or 2×10^4 molecules of each amplicon per reaction and used non-targeted tree DNA for the negative control. Positive controls showed that primers amplified the intended targets and negative controls were checked to help identify the read-cut-off calculation. Any reads showing up in the negative control may be the result of index hopping, sequencing error in the indices or contamination. As a rule, we set a minimum read threshold of five reads, or used the negative control to calculate a 95th percentile minimum read threshold (using whichever number is greater). Reads at or below this threshold were removed from the data set. Counts above the threshold were considered 'positive hits' for that

taxon in that sample × primer combination. For this data set, we used 5 as the minimum read threshold.

Target amplification was performed by the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center using a Fluidigm 48.48 Access Array and using the two-step Fluidigm cycling parameters with a modified annealing temperature of 58°C. Traditional bias in a multiplex PCR reaction, in which one primer out-competes others and dominates the reaction, does not apply in the Fluidigm platform because each primer and sample combination are performed in separate reactions. For example, the Fluidigm is a microfluidic platform with very small nano-chambers that carries out each primer pair reaction individually in a 48 samples×48 individual primer pairs grid per array for a total of 3204 individual reactions. The resulting barcoded products are then mixed together in equal volumes prior to sequencing. Bovine serum albumin (BSA) was added at 0.2 μg μL^{-1} final volume to mitigate PCR inhibition. Amplified targets were then sequenced on an Illumina MiSeq (Ilumina, San Diego, CA, USA) with 2x250 V2 chemistry.

Sequences were returned from the vendor demultiplexed by index and by primer using a custom pipeline they designed to sort Fluidigm data (see raw data in Table S2). For this run, the pipeline allowed one mismatch in the index and two mismatches in the primer. Owing to anomalies found in the read 2 data during joining of the paired-end sequences, only read 1 sequence was used in subsequent analyses. Trimming of the read 1 sequence was done in primer groups using Trimmomatic v. 0.33. Using amplicon-specific information, the 5′ end of the read was trimmed the length of the primer plus 5 nucleotides and the overall length of the read was cut-off at 200 bp to avoid analysing the 3′ end of the sequence where quality scoring of read 1 sequencing began to drop off.

Following demultiplexing and trimming, the reads were classified to taxon using the programs KMA, version 1.2.23 (Clausen et al., 2018), and CC-Metagen version 1.2.2 (Marcelino et al., 2020) and the Marcelino indexed database dated 11 June 2019, which includes all sequences from the NCBI nucleotide collection except environmental or artificial sequences (scripts in Table S3). The KMA/CC-Metagen pipeline generates a consensus sequence from all sequences that match a kmer hit and each other. As a result, there are several consensus sequences that are of the same species, but different haplotypes, and if there is variation that is captured and meets the tested threshold, it will be retained in a final sequence. Random sequencing errors that occur in low rates are smoothed out by this process and not represented in the final consensus sequence that is recorded and compared against the database. It is possible that if an error is incorporated early in the amplification process, it will be retained in the data set as it will occur in higher abundance, but that would be a very unusual event and true sequence information will dominate in the final analysed sequences. As our data consist of many different gene targets of varying conservation, length and taxonomic origin, we needed to modify the thresholds to better capture species richness. We found the following taxon threshold settings best fit this particular data set: class = 79.0, order = 80.0, family = 84.0, genus = 91.4 and species = 94.4. When setting the CC-Metagen genus and species thresholds, we used our positive control to guide the parameters because we know which templates were in the control. The selected parameters provided the most accurate identities possible in the positive control sequences across all genes and species targeted. For each site, the number of reads for each sample × primer combination was counted and summed.

2.4 | Data analyses

To examine the presence and read abundance of eDNA surveys for all analyses, we summarized read counts above the minimum read threshold for each site for all replicate × primer combinations for targeted taxa. A visualization of all targeted taxa detected for each site was created with Krona (version 2.7; Ondov et al., 2011). For subsequent analyses, we focused on a subset of the broadly targeted biota including detected fishes, amphibians, crayfishes, mammals and one amphibian pathogen, except for the comparison of electrofishing surveys to eDNA where we focused on fishes and amphibians.

We used nonmetric multidimensional scaling (NMDS; 9999 random starts), a nonparametric ordination technique, to visualize biodiversity assemblages including the number of reads for focal taxa by site, watershed, above or below fish distributions, and position of watershed in the basin. NMDS, an iterative process that seeks to minimize stress of a k-dimensional figure, was conducted to explain the similarities among assemblages. To calculate the similarity matrix, we used the square-root transformation of the Euclidean distance among the number of reads per taxa to reduce the highly influential taxa at each site (Clark, 1993; McCune & Grace, 2002). The resulting matrix was 32 rows (4 watersheds × 8 sites) × 36 taxa (columns) for a total of 1152 values. We added ellipses based on the flexible beta cluster to help identify patterns in how the data cluster around the distribution limit of fishes. For the NMDS analyses, we used the software Primer7 (United Kingdom; Clarke and Gorley (2015)).

To understand whether samples vary significantly between sites in the fish-bearing portion of streams and above that point or whether there is gradual change along the stream continuum not influenced by fish occupancy, we used PERMANOVA. We also performed a permutation test for homogeneity of dispersion of multivariate analyses (PERMDISP), which allows us to test the dispersion of assemblage distances between sites above or below the fish distribution boundary. These community analyses allowed us to evaluate whether species of all taxa drop out gradually along an upstream–downstream gradient (which would be shown by a lack of significance in the PERMANOVA analysis and greater dispersion from the centroid across taxa) or whether they drop out abruptly at a jump or critical point (which would be supported by PERMANOVA significance and a tight dispersion around the centroid). For the PERMANOVA and PERMDISP analyses, we used the community ecology R package vegan 2.6–5 Oksanen et al. (2022).

Change in species assemblages from beta diversity, which measures the extent of change in species assemblages, can result from either nestedness or turnover (Baselga, 2010). We examined beta diversity within streams and for sites above and below fish where Sorensen dissimilarity ($ß_{SOR}$) is partitioned between turnover ($ß_{SIM}$) and nestedness ($ß_{SNE}$;

Baselga, 2010). We identified whether differences in assemblages are driven by species loss from site to site (nestedness) or by the replacement of species between sites (turnover). For the beta diversity analyses, we used the R package betapart 1.5.6 (Baselga & Orme, 2012).

We used rarefaction analysis to understand the effects of variable assemblage composition, sample size, spatial sampling effort and our bioinformatics criteria have on the species richness. We estimated species richness with the Chao II bias-corrected estimator using a rarefaction analysis of 9999 randomization (Evans et al., 2017). For the rarefaction analysis, we used the software Primer7 (United Kingdom; Clarke and Gorley (2015)).

3 | RESULTS

3.1 | eDNA analyses

After bioinformatic analysis and minimum read threshold filtering, we identified 2,085,527 reads to taxon, including reads from primers targeting fishes, amphibians, crayfishes, freshwater insects, bivalves, mammals, fungi and forest and animal pathogens (Figure S1; Table S2). Depending on the watershed or site, there are different proportions of taxon that contribute to detections and number of reads. For example, oomycetes comprise of 49% of the total reads at Gus Creek and 51% at Rock Creek, whereas they are 23% at Upper Mainstem Trask River and 24% at Pothole Creek.

For our analyses, we took a closer look at 1,140,565 reads from the four neighbouring watersheds composed of detected fishes, amphibians, a crayfish, a mammal and an amphibian pathogen (Figure 3, Table S4). The largest number of reads came from Pothole Creek (428,555) and upper mainstem Trask River (385,465), with fewer reads from Rock Creek (52,657) and Gus Creek (90,881). We did not detect bivalves, including mussels or clams, at any site. Some taxa had low read counts but were above our threshold and are likely misclassifications, as these fishes do not exist in the Trask River watershed, including *O. gilae*, *O. masou* and *Cottus* reads from other continents (Table S4). Consequently, we left *O. gilae* and *O. masou*, out of our analyses and grouped all Sculpin lineages from other continents into one taxon of *Cottus* spp. to represent sculpins that need

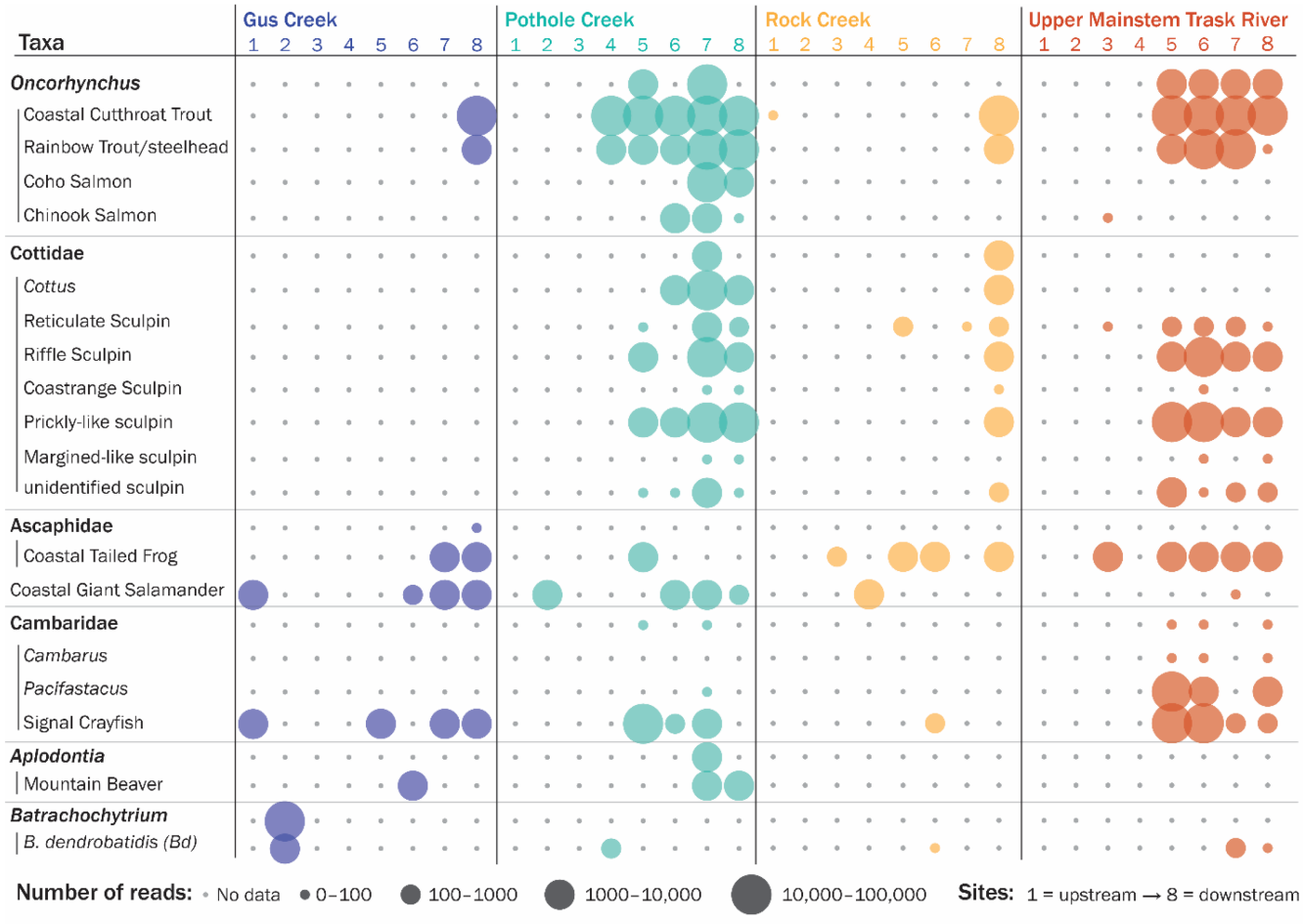

FIGURE 2 Detections of taxa by eDNA shown as a coloured bubble across sites in four neighbouring watersheds, including Gus Creek (blue), Pothole Creek (green), Rock Creek (yellow), upper mainstem Trask River (dark orange) watersheds. Size of the bubbles represents eDNA relative abundance based on number of reads on a log scale with actual values found in Table S1. No detections are represented as a grey dot. The site furthest upstream is referred to as '1' and the furthest downstream site is '8'.

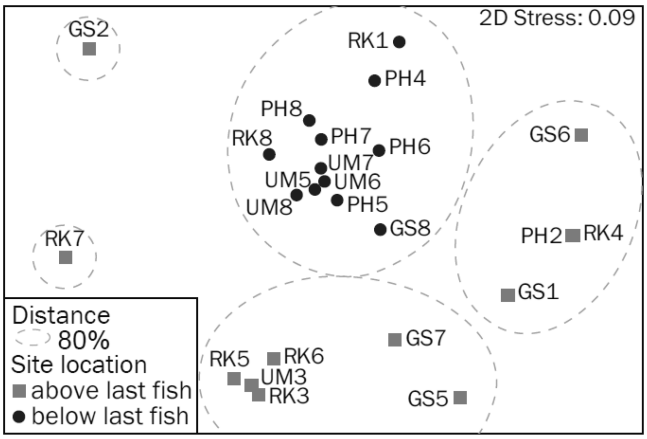

FIGURE 3 Nonmetric multidimensional scaling ordination of eDNA biodiversity assemblage across study sites in Gus Creek (GS), Pothole Creek (PH), Rock Creek (RK) and upper mainstem Trask River (UM) watersheds coded by whether the site was above (blue squares) or below (dark orange circles) fish distributions owing to eDNA results. The number of reads per taxa by site can be found in Table S1. The distance between symbols reflects their degree of similarity (higher similarity = closer together, more dissimilar = further apart).

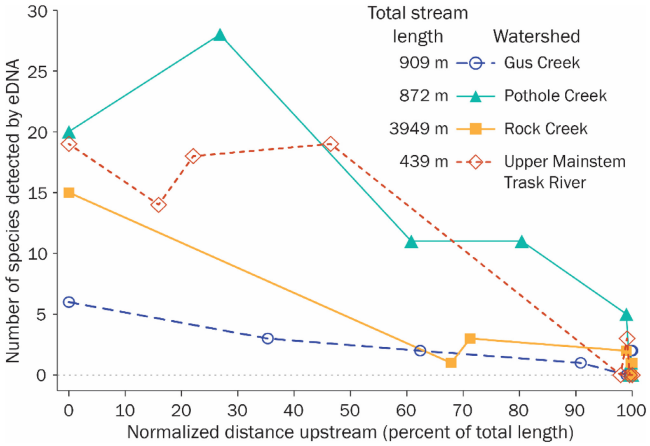

FIGURE 4 Species richness from eDNA by stream distance (normalized; distance between two points/total stream distance × 100) across sites in Gus Creek, Pothole Creek, Rock Creek and upper mainstem Trask River watersheds. Sites 1–8 are noted with symbols across watersheds, including Gus Creek (blue circle), Pothole Creek (green triangle), Rock Creek (yellow square) and upper mainstem Trask River (dark orange diamond) with site 8 starting at 0.

further investigation. The rest of the *Cottus* reads mapped to recognized species.

Multigene eDNA metabarcoding allowed us to detect a shift in the freshwater assemblages as we moved upstream, revealing a spatial continuum of species replacements from fishes no longer being detected to a continuation of amphibians (Figure 2, Table S4). Downstream sites yielded more species and DNA signal compared to upstream sites. Species composition is significantly affected by whether a site is above or below fish (PERMANOVA $R^2 = 0.46$, pseudo p-value <0.001). Sites below or within fish distributions had species assemblages that were more similar to one another than sites above fish, marking a distinct ecological change (Figure 3). Sites below fish separate into their own single cluster distinct from sites above fish that group into multiple clusters highlighted by ellipses based on flexible beta clusters (cophenetic correlation 0.93, optimum beta 0.02). Along this same line of inquiry, sites above the fish distribution boundary have a broader dispersion than sites below fish, which have a tight dispersion (PERMDISP, $F = 9.09$, pseudo p-value <0.001). In some instances, within and across watersheds, however, some species had patchy distributions. For example, Signal Crayfish *Pacifastacus leniusculus* were detected by eDNA in the fish part of the network and were also detected upstream in Gus Creek. More taxa were detected downstream than upstream across watersheds (Figure 4). There was also lower signal detected across species at upstream sites, except for Coastal Giant Salamander, *Dicamptodon tenebrosus*, which had their highest signal at an upstream site in most watersheds (Figure 4, Table S4).

Turnover or species replacement is the main mechanism underlying beta diversity or species richness across and within watersheds (Table 1). Across watershed turnover is the main cause of changes in species richness, although nestedness or species loss is relatively high in Pothole Creek and at sites above fish likely also playing a smaller, but important role at those sites. Gus and Rock Creeks have the highest spatial turnover among the four watersheds. Along a stream continuum or gradient within watersheds, there are changes in species composition where fish drop out mainly owing to turnover.

Our results reveal that freshwater biodiversity across all watersheds can be comprehensively inventoried from three samples using multiple primer sets, although specific watersheds need a different number of samples to comprehensively evaluate their biodiversity (Figure 5). Specifically, Pothole Creek and Upper Mainstem Trask River plateaued after two samples, whereas Gus Creek plateaued around eight samples and Rock Creek plateaued closer to 12 samples.

Coastal Cutthroat Trout, sculpins, steelhead and Signal Crayfish *Pacifastacus leniusculus* were the most common taxa detected by eDNA across watersheds (Figure 2, Table S4). The eDNA sampling also detected species, such as the amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*), in all sites; the Mountain Beaver, *Aplodontia rufa*, in Gus and Pothole creeks; and Chinook Salmon *O. tshawytscha* in Pothole Creek and Upper Mainstem Trask River. Across watersheds, we found Pothole Creek had higher biodiversity for species richness and eDNA number of reads relative to the other three watersheds.

3.2 | eDNA versus electrofishing

For fishes and amphibians, multigene eDNA metabarcoding and electrofishing detected from 5 to 12 taxa at the downstream-most site depending on the watershed (Figure 6). The eDNA methods detected more taxa across all watersheds relative to electrofishing, except in Gus Creek where both methods detected a total of five

TABLE 1 Beta diversity metrics, which measure dissimilarity in assemblages, including Sorensen dissimilarity, turnover (species replacement) and nestedness (species loss) in four neighbouring watersheds of the Trask River, including both above and below fish.

	Sorensen dissimilarity ($ß_{SOR}$)	Turnover ($ß_{SIM}$)	Nestedness ($ß_{SNE}$)
Gus Creek	0.79	0.65	0.14
Pothole Creek	0.73	0.38	0.35
Rock Creek	0.91	0.71	0.19
Upper Mainstem Trask River	0.54	0.29	0.25
Above fish	0.84	0.50	0.34
Below fish	0.87	0.59	0.28

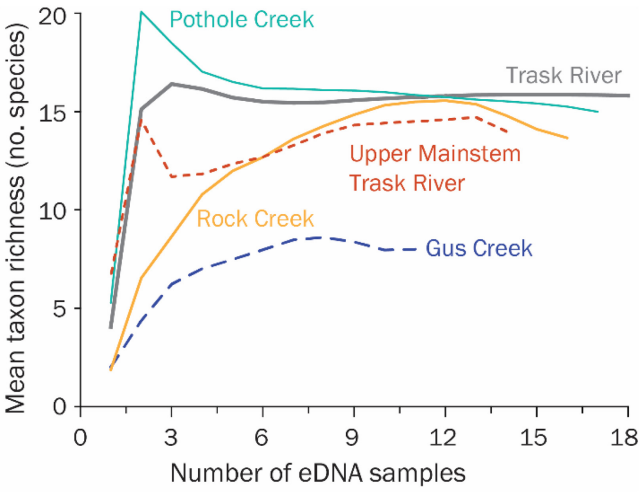

FIGURE 5 Number of species as a function of the number of eDNA samples across watersheds, including Gus Creek (blue), Pothole Creek (green), Rock Creek (yellow) and upper mainstem Trask River (dark orange) and for the whole Trask River (grey). The number of eDNA samples is pooled across replicates and sites for each watershed.

fish, amphibian or crayfish taxa, although not the same five species. Electrofishing and eDNA detected Coastal Tailed Frog and Coastal Giant Salamander across watersheds.

The eDNA methods had the added benefit of detecting additional groups of sculpins that mapped to other recognized sculpins but are not known to be in the Trask River watershed, or that could not be assigned to any recognized species, both of which suggest the presence of more sculpins than expected based on electrofishing, including apparent cryptic lineages (Figure 6; Table S4). Although taxonomic identifications were confirmed from voucher specimens following electrofishing surveys describing Reticulate Sculpin in all streams and Torrent Sculpin in Rock Creek, eDNA surveys based on genetic classifications proposed many more potential lineages of sculpins. For example, additional classifications revealed assignments that mapped to recognized sculpins in the region, such as Prickly, Margined, Riffle or Coastrange Sculpins, along with

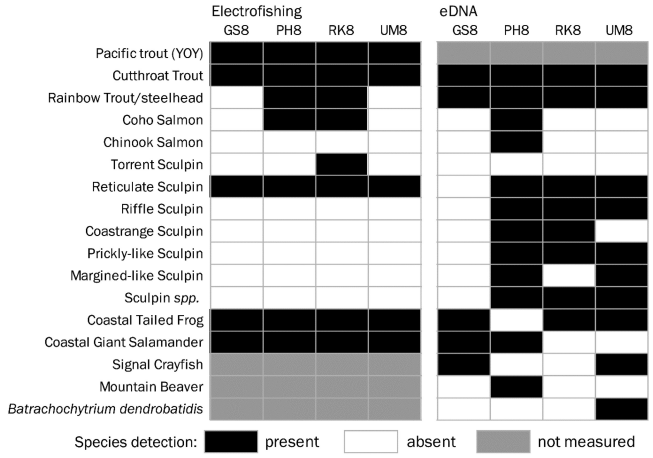

FIGURE 6 Detections of taxa from eDNA and electrofishing for Gus Creek (GS), Rock Creek (RK), Pothole Creek (PH) and upper mainstem Trask River (UM) watersheds from the most downstream site (site 8) that is nearest to the confluence with Trask River. Presence (black), absence (white) and not measured (grey) are shown for both electrofishing and eDNA methods.

assignments to sculpins from other regions and even continents. Collectively, this suggests additional sculpins in the Trask River watershed and apparent cryptic species.

The eDNA results revealed discrimination in YOY Pacific trout (both *O. mykiss* and *O. clarkii clarkii*), which is not possible without genetic methods to distinguish them (Figure 6). For example, steelhead *O. mykiss* were detected at all downstream sites, instead of two as suggested by electrofishing methods. Pacific trout were detected further upstream with eDNA than expected based on previous electrofishing efforts.

4 | DISCUSSION

We reveal a compelling range of biodiversity in stream waters across a broad array of taxa, thereby expanding our understanding of species presence, species distributions and biodiversity. Our results detect a shift in the freshwater assemblage where fish drop out in streams, with fewer species at lower signal upstream, except for Coastal Giant Salamander. Our results show rare taxa, apparent cryptic lineages of sculpin, and refined distributions for salmonids, which provides the basis for identifying patterns of biodiversity and is the first step in beginning to identify which species can adapt to change. Collectively, we show evidence for extensive diversity in streams, some of which was previously hidden, which affects our understanding of species and our ability to track, protect and manage them across watersheds.

4.1 | Shift in the freshwater assemblage at the fish distribution limit

We discovered a shift in the freshwater assemblage at the fish distribution limit, with fewer species and reduced eDNA signal

upstream, suggesting that a major shift in freshwater biodiversity occurs as a detectable jump when fish drop out of streams. This finding supports the hypothesis that the boundary delimiting the upper extent of fish marks a distinct ecological change in freshwater biodiversity assemblages and may be the nexus in the stream networks that unifies stream concepts. The usual upstream–downstream gradient or stream continuum suggested by the river continuum concept is supported below the fish distribution boundary with similar assemblages and a tighter assemblage dispersion. However, the boundary at the upper extent of fish marks a jump in freshwater biodiversity through a loss of fishes from species turnover, implying that there is spatial structure for stream assemblages around the limit of fish. The upper extent of fish marks a distinct geophysical stream point above which no more fishes are found owing to specific abiotic factors that collectively intersect to abruptly limit fish (Penaluna et al., 2022). Species turnover has been found in other studies looking at fish distributions along a stream continuum (Li et al., 2018; Nakagawa et al., 2018). Collectively, our results highlight the ecological importance of the upper extent of fish to freshwater biodiversity assemblages owing to the discrete loss of fish and some co-occurring species.

The number of detected species declines with progression upstream in all four neighbouring watersheds, similar to Li et al. (2018), suggesting that smaller headwater stream reaches support fewer species and at lower numbers than medium-sized stream reaches located downstream. Habitat diversity and environmental stability increase downstream, which also coincides with the downstream accumulation of species (Tornwall et al., 2015). Coastal Giant Salamanders are the exception, with their highest read count at an upstream site in most watersheds. Higher read counts upstream may be from less competition from fishes. Alternatively, it could be from potentially larger substrate sizes in those stream reaches, which is related to increased Coastal Giant Salamander biomass density (Rundio & Olson, 2003).

We find distinct assemblages of species from site-to-site within these neighbouring watersheds likely owing to actual heterogeneous biodiversity. More generally, eDNA is not uniformly transported downstream owing to physical and biological degradation in streams (Shogren et al., 2017), which our results reveal in the patchy detection of taxa between sites along a stream continuum, especially sparsely detected taxa, suggesting that eDNA signal is not saturating far downstream of its source. Rather, most eDNA signal settles into the local environment, such as the benthic substrate, as it continues to decay (Harrison et al., 2019; Turner et al., 2015). This result proposes that the downstream transport of eDNA is insufficient in these watersheds to homogenize the signal of species diversity from upstream sites at downstream sites, similar to Li et al. (2018). It is likely that the transport distance in these watersheds is less than the between-site distances during seasonal low flow. Our results reveal the distribution of stream vertebrates is highly dynamic, with individuals distributing patchily at the fine scale.

4.2 | Hidden biodiversity in streams

We reveal a hidden world of diversity in streams, including the amphibian pathogen *Bd*, the native Mountain Beaver, Chinook Salmon and the apparent absence of freshwater mussels and clams. These taxa have been under-surveyed or undetected with other methods. Another study found three more species using eDNA metabarcoding that were undetected by electrofishing (Olds et al., 2016). Understanding the full scope of biodiversity can be improved using additional barcodes (McColl-Gausden et al., 2021) and multiple sets of universal primers (Stat et al., 2017) as we have done here.

The amphibian pathogen *Bd* is likely more common in streams than previously understood as it was detected in 49% of sampled watersheds (Olson et al., 2021) and across all watersheds in this study, although sparsely distributed. Mountain Beaver are not normally assessed in stream surveys and eDNA offers an opportunity to delineate their distribution. Chinook Salmon have not been detected this high-up in these watersheds with traditional sampling of backpack electrofishing (Jensen, 2017). Underestimation of uncommon or rare species leads to an underestimation of biodiversity. Thus, more accurate and complete biodiversity assessments that include rare and uncommonly surveyed species lead to a better understanding of biodiversity, with consequences for species conservation.

Rare taxa have low population densities and can be sparsely distributed, increasing the probability of imperfect detections. The richness of such uncommon taxa is an important proportion of overall biodiversity, accounting for 14% or less of species across watersheds here. A study using eDNA metabarcoding shows that rare species may be better detected using more than one gene (12S and cytochrome b; Lawson Handley et al., 2019). Other studies have also found high sensitivity with eDNA using qPCR platforms targeting rare freshwater species, including trout (Wilcox et al., 2013), sturgeon (Pfleger et al., 2016), eels (Itakura et al., 2019) and stream amphibians (Pilliod et al., 2013). Rare species often contribute to ecosystem services through species interactions, such as contributing to community stability (Zatkos et al., 2021), emphasizing their importance to the ecosystem (Dee et al., 2019).

4.3 | Uncovering additional diversity for sculpins

We uncover more lineages of sculpins with eDNA metabarcoding than expected from electrofishing, including apparent cryptic lineages and possibly a newly discovered cryptic species complex(es). Our eDNA results show detecting many more sculpins than previously expected through electrofishing, which could have ramifications for potential pockets of endemism, the possible identification of new species and management strategies for conservation of fishes. Cryptic species are distinct species that have incorrectly been classified as a single species. Cryptic species complexes often eventually resolve into multiple species with low abundances and small distributions, making them conservation priorities. In some cases, cryptic species may represent the early evolutionary stages after speciation

(Des Roches et al., 2021). Sculpins have been shown to contain cryptic taxa and multiple lineages within groups (Baumsteiger et al., 2012; Young et al., 2013, 2022). In the Trask River watershed, which is a Pacific coastal watershed, there are at least two main groups of sculpins, including the Coastrange Sculpin *C. aleuticus* species complex (a strictly coastal, freshwater species complex found lower down in watersheds) and the Prickly Sculpin *C. asper* species complex, which consists of at least five recognized species (Baumsteiger, 2013; Page & Burr, 2011; Young et al., 2022). However, Reticulate Sculpin *C. perplexus* are not considered to be part of either of those species complexes and may represent a distinct species complex. We posit that new genomic methods using eDNA are a promising tool to reveal the hidden variation within and among species to better identify cryptic species and understand the ability of each species to cope with change. Our current taxonomic framework for sculpins is incomplete, and consequently, future work could focus on distinguishing lineages and identifying novel, unnamed lineages within these cryptic sculpin complexes by defining taxon boundaries.

4.4 | Distribution extensions for Pacific salmon and trout

Our eDNA results reveal a distribution extension for Pacific salmon and trout, effectively extending the upper limits of fish in streams (Figure 2; Table S1). We genetically distinguish individuals of morphologically similar species earlier in their development (YOY) than is possible by field identification. Coastal Cutthroat Trout are detected further upstream with eDNA than expected based on previous electrofishing across watersheds, as also found by Hauck et al. (2019) and Penaluna et al. (2021). Watersheds in coastal Oregon, where the Trask watershed is located, contain the greatest diversity for Coastal Cutthroat Trout, harbouring unique haplotypes (Weitemier et al., 2021). Coho and Chinook salmon and steelhead, all likely juveniles, are also detected higher in the watershed in Pothole Creek, suggesting an upstream extension of their distribution. Detecting species with eDNA metabarcoding is more sensitive than traditional methods (Hauck et al., 2019; Olds et al., 2016; Valentini et al., 2016; Yamamoto et al., 2017). For example, eDNA metabarcoding can offer a more efficient biodiversity assessment of streams over a longer stream section than traditional methods that survey relatively smaller stream reaches (Civade et al., 2016). Collectively, these distribution results suggest that eDNA reflects a here-and-now presence of species. Given that the upper extent of fish distributions has significant implications for species conservation and forest management, comprehensive biodiversity assessments could help to reinvent the management of these systems and help meet multi-management objectives.

4.5 | Future work and study limitations

Although multigene eDNA metabarcoding reveals a world of hidden biodiversity in freshwaters, more research is needed to decipher sources of error in the metabarcoding process that create difficulty distinguishing erroneous detections from real detections of rare or recently introduced species. Calculating a read threshold from negative control samples allows us to account for noise from contamination or where reads from one sample show up in another (e.g. barcode swaps), but it does not account for sequencing or database noise for taxa that are only differentiated by a few single nucleotide polymorphisms (SNPs). Such errors may account for the detection of, for example, *O. gilae*, *O. masou* and some of the *Cottus* reads, which have low read counts, but above our threshold (Table S4). Fewer misclassification errors would also occur if there were a regional or local database linking vouchered specimens to genetic information. Low read counts affect species detections, suggesting the importance of taking more field samples and replicates. Negative field controls during fieldwork also would allow for the ability to discard cross-contamination between sites. Further research into sculpin taxonomy and lineages would help to identify how many sculpin species or complexes exist in the Trask River watershed and determine whether there are new species and/or potential pockets of endemism there. Understanding freshwater species characteristics and life-history traits that affect eDNA detection are key (Evans et al., 2016) to comprehensively sample biodiversity.

5 | CONCLUSIONS

This study enhances the biogeographic understanding of freshwater biodiversity and unifies stream concepts by showing that both concepts exist in the distribution of taxa near the distribution limit of fish. We reveal evidence for extensive freshwater diversity, some of which was previously hidden, that affects our understanding of species and assemblages. We detect overlooked and underappreciated organisms alongside species that are common or have elevated levels of protection. Newly detected cryptic lineages in streams present opportunities to study speciation and reevaluate conservation management. A relatively full freshwater biodiversity assessment, such as this, helps to identify appropriate units of conservation for species and assemblages, offers a first step in understanding each species' role as an intrinsic part of biodiversity and provides a baseline for understanding change in species distributions over time. Ultimately, we reveal the spatial structure in stream biodiversity around the uppermost boundary of fish and propose that other portions of stream networks may also have important spatial structuring.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

DATA ARCHIVE

All raw data associated with this study have been archived in the NCBI's Sequence Read Archive PRJNA852368.

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BIOSKETCH

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Author Contributions: BEP, RC and TSG conceived the study; BEP and RC developed field protocol; LLH designed eDNA primers and ran data through pipeline; BEP analysed the data; BEP wrote the original draft; BEP, RC, LLH, KAW and TSG reviewed and edited the manuscript.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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